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VITAMIN B_{12} COMPOUNDS IN RELATION TO THE REQUIREMENTS OF COBALT FOR HIGHER PLANTS AND NITROGEN-FIXING ORGANISMS*

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Most investigators agree that higher plants contain little or, in most cases, no detectable vitamin B_{12} compounds† in their tissues. This point was emphasized by the discovery by scientists in Australia and New Zealand that cattle and sheep grazing on certain pastures suffered from a severe deficiency disease that could be cured by a supply of cobalt or cyanocobalamin. The initial investigations,[1,2] beginning in 1934, and more recent developments that have contributed to an understanding of the problem, were reviewed by Marston,[3] Underwood,[4,5] Young,[6] and Johnson and Bentley.[7] From these discussions it is apparent that certain soils in Australia, New Zealand, Britain, Canada, Eire, Germany, Holland, Kenya, Poland, South Africa, Sweden, The United States, and Soviet Russia contain insufficient cobalt to meet the needs of ruminant animals. A few cases of cobalt deficiency have been observed in nonruminant domestic animals, but the incidence of the disease is much more prevalent in ruminants. The application of cobalt salts to deficient soils, or the addition of cobalt to the feed to deficient ruminant animals, either prevented the symptoms or cured deficient animals. The injection of inorganic cobalt salts into deficient animals, however, failed to affect the deficiency symptoms, but the injection of cyanocobalamin or the feeding of massive doses of cyanocobalamin alleviated the deficiency symptoms. These and other investigations have led to the conclusion that pasture plants contain insufficient concentrations of B_{12} compounds to meet the needs of ruminants. If there is sufficient cobalt in the plant materials ingested by ruminants, bacteria in the rumen synthesize vitamin B_{12} compounds and these are utilized in the metabolism of both the animal and the bacteria.[7,4,5]

The conclusions drawn by Ford and Hutner,[8] Hutner *et al.*,[9] and Young[6] are consistent with those of the Australian and New Zealand scientists and indicate that there is a lack of conclusive evidence that higher plants can synthesize vitamin B_{12} compounds. Experimental evidence for this conclusion was obtained by use of a variety of microorganisms for the biological assay of B_{12} compounds. Ford and Hutner[8] and

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† The general term "vitamin B_{12} compound" refers to any member of the vitamin B_{12} family. Where a specific compound is known, the specific name is used, *i.e.*, "cyanocobalamin."

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Ford[10] have pointed out that assay organisms vary greatly in their response to B_{12} compounds. As a result, in most cases the identity of the B_{12} compounds detected in plant materials has not been established.

In 1950, Robbins *et al.*[11] used *Euglena gracilis* as an assay organism to investigate the B_{12} compounds in a series of higher plants. Their results showed that roots contained low concentrations, and leaves were devoid of the vitamin. In their opinion, the vitamin B_{12} compounds found in roots very likely were synthesized by microorganisms from the soil. Bickoff *et al.*[12] measured the content of B_{12} compounds in alfalfa by use of *Lactobacillus leichmannii* as an assay organism and found that fresh material contained concentrations ranging between 12 and 45 ppm. Further investigation provided evidence that at least 85 per cent of the B_{12} compounds detected in their assay could be accounted for by substances other than the "true vitamin." They suggested that deoxyribosides may have been responsible for a portion of the apparent B_{12} activity. In another study, Gray and Daniel[13] detected vitamin B_{12} in a sample of turnip greens from one location in Georgia, but they were unable to identify the vitamin in a sample from another location in the same area. A critical examination of their samples was made by use of paper electrophoresis, paper chromatography, and *Ochromonas malhamensis* as an assay organism. Convincing evidence was presented showing that the properties of a compound isolated from one sample of turnip greens were essentially the same as those of cyanocobalamin. Since this or other B_{12} compounds were not consistently identified in other samples of turnip greens, Gray and Daniel suspected that the vitamin found in one sample was derived from microbial sources.

If higher plants synthesize and utilize vitamin B_{12} compounds in necessary metabolic processes, it follows that cobalt is a necessary micronutrient element for growth. Several individuals[14-17] have reported that the application of small amounts of cobalt to experimental plots in fields, or to plants grown in sand or solution cultures, resulted in increased yields of plant material. In those cases where critical examinations were made of the cobalt requirements of higher plants grown in absence of symbiotic microorganisms, inconclusive or negative results were reported. One of the most careful series of experiments designed to test the possibility that nonleguminous higher plants require cobalt was conducted in Malaya by Bolle-Jones and Mallikarjuneswara.[14,18] They cultured rubber (*Hevea brasiliensis*) and tomato (*Lycopersicum esculentum*) plants in purified sand using highly purified nutrient solutions and demonstrated a small but statistically significant increase in growth of both species from the addition of cobalt. Lack of cobalt, however, caused no visual symptoms of deficiency or changes in the content of chlorophyll or of major mineral components in tissues. As a result, the essentiality of cobalt for these species was not claimed. One is forced to the conclusion, therefore, that no compelling evidence has been provided that higher plants require cobalt for growth or synthesize vitamin B_{12} compounds.

Recently it has become apparent that not only the microorganisms that

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live in symbiotic association with ruminant animals, but also those that participate in a symbiotic relationship with leguminous and nonleguminous nitrogen-fixing species, synthesize vitamin B_{12} compounds provided that the cobalt supply is adequate. In addition, considerable information has accumulated showing that certain algae and nitrogen-fixing bacteria synthesize vitamin B_{12} compounds and require cobalt for growth. In the remainder of this paper, therefore, emphasis will be given to the occurrence of vitamin B_{12} compounds in certain algae, bacteria, and nitrogen-fixing symbionts in relation to the cobalt requirements of these organisms.

Vitamin B_{12} Compounds and Cobalt in the Nutrition of Algae

Certain algal species synthesize vitamin B_{12} compounds and therefore require traces of cobalt in media used for their culture. A need for cobalt has been established only for certain blue-green species that are known to fix atmospheric nitrogen.[19] Only minute traces of the element are needed for these organisms and, therefore, components of nutrient media had to be rigorously purified before consistent responses to cobalt additions were obtained. The first demonstration of a cobalt requirement for blue-green algae was made by Holm-Hansen *et al.*[20] in 1954. They cultured *Nostoc muscorum, Calothrix parietina, Coccochloris peniocystis,* and *Diplocystis aeruginosa* (*Anacystis cyanea*) in a dilute nutrient solution in order to avoid excessive impurities and showed that 0.4 μg. of cobalt per liter was essential for optimum growth. Significant increases in the nitrogen content of cells was obtained with a concentration of 0.002 μg. of cobalt per liter. The cobalt requirement of these algae was satisfied by cyanocobalamin. The significant observation was made by Holm-Hansen and coworkers[20] that those species of blue-green algae capable of fixing atmospheric nitrogen exhibited a greater response to cobalt than those requiring combined forms of nitrogen. On the basis of this observation, it was suggested that cobalt may function in the nitrogen-fixing process.

In another investigation, Okuda and Yamaguchi[21] isolated a blue-green alga, *Tolypothrix tenius,* from a paddy soil in Japan and reported that the addition of cobalt salts to the culture medium stimulated both growth and the synthesis of vitamin B_{12} compounds. *Lactobacillus leichmannii* and *Escherichia coli* (strain 113–3) were used by these investigators to detect B_{12} active substances in the experimental materials. In their experiments, the addition of 0.2 mg. of $CoCl_2 \cdot 6H_2O$ per liter of medium increased the dry weight of *T. tenius* cells about 15 per cent and the content of B_{12} compounds about 17 per cent in comparison with control cultures. They concluded that the capacity of *T. tenius* to synthesize vitamin B_{12} compounds was not related to the nitrogen-fixing capacity of the organism. It should be pointed out, however, that the components of the nutrient media in these experiments were not purified and, therefore, control cultures lacking added cobalt undoubtedly contained appreciable concentrations of the element.

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Perhaps the most definitive research on the cobalt requirements of blue-green algae was carried out by Buddhari[22] in D. I. Arnon's laboratory in California. By use of a copper sulfide coprecipitation procedure and dithizone extraction to remove traces of cobalt from the nutrient media, Buddhari established beyond any question that species of *Anabaena* and *Nostoc* needed traces of cobalt for growth. The cobalt requirement was highly specific since no other element in a series tested would replace it. Cobalt added in the form of cyanocobalamin, however, was more effective at low concentrations than an equal amount of the element in the form of inorganic cobalt salts. The traces of cobalt supplied to *Anabaena* and *Nostoc* species were converted into vitamin B_{12} compounds, but only a small portion of these were in the form of the "true vitamin B_{12}." The cobalt requirement of *Anabaena* sp. was determined in media supplied with different nitrogen sources and, in agreement with the reports of Okuda and Yamaguchi,[21] the quantity of the element needed for growth was not influenced by the form of nitrogen supplied.

Many algal species lack the capacity to synthesize B_{12} compounds and, therefore, must be supplied with an exogenous source of the vitamin. In other species, B_{12} compounds have been detected by biological assay procedures but, with the exception of those species already mentioned, cobalt requirements have not been demonstrated. The role of trace elements, including cobalt, and the role of vitamins in the growth of algae has been discussed from several aspects by Provasoli,[23,24] Hutner *et al.*,[9,25] Droop,[26] and Weissner.[27] Further consideration of this subject will be limited, therefore, to some examples where B_{12} compounds have been identified in algae or where exogenous B_{12} requirements have been established. Most of the species of algae to be mentioned have not been shown to fix atmospheric nitrogen but a brief consideration of the role of B_{12} compounds in different classes of algae seems appropriate.

According to Pintner and Provasoli,[28] an exogenous source of vitamin B_{12} compounds is necessary for growth of the red pigmented blue-green alga, *Phormidium persicinum*. Any one of the cobalamin compounds substituted for cyanocobalamin but methionine, deoxyribosides, or inorganic cobalt salts were ineffective. Another blue-green alga (*Synechocystis*) also is reported[29] to have an absolute requirement for vitamin B_{12}. In the culture of this organism, either cyanocobalamin or any one of a series of B_{12} analogues functioned satisfactorily as a growth factor. In this report, Van Baalen[29] pointed out that over 50 per cent of the marine blue-green algae recently isolated in his laboratory required vitamin B_{12} compounds in the media used for their culture. Bacteria-free cultures of *Plectonema nostocorum, Calothrix parietina, Aphanizomenon flosaquae, Diplocystis aeruginosa* (*Anacystis cyanea*) all produce compounds active in the *Euglena gracilis* assay for vitamin B_{12} (Robbins).[11] In addition, vitamin B_{12} compounds have been detected in *Anabaena cylindrica* (Fogg[19]). It seems apparent that the vitamin is widely distributed in or is required for most of the blue-green species.

Buddhari,[22] Droop,[26] and Provasoli[23,24] have pointed out that certain

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algae other than the blue-green species either require or synthesize B_{12} compounds. The green alga *Lobomonas rostrata* and also *Stichococcus* species synthesize B_{12} compounds according to Lewin.[30,31] *Euglena gracilis* and *Ochromonas malhamensis* require exogenous B_{12} for growth and are often used for assay organisms. The specificity of the response of these organisms to various B_{12} compounds was discussed by Robbins *et al.*[32] and Ford.[10] Brown and coworkers[33] have detected B_{12} compounds in the green alga, *Chlorella vulgaris*, and Provasoli and Pintner[34] have demonstrated that algal flagellates including *Peridinium* sp., *Cyanophora paradoxa*, *Synura* sp., and a marine *Gyrodinium* require cyanocobalamin for growth. In a comprehensive summary of the vitamin requirements of algae, Provasoli[23] has cited evidence showing that five species of Chlorophyceae, nine species of Euglenineae, four species of Cryptophyceae, and three species of Bacillariophyceae require cyanocobalamin for growth.

No convincing evidence has been published proving that algae other than certain blue-green species require cobalt as an essential micronutrient element. With the exception of the observations of Holm-Hansen *et al.*,[20] no evidence is available showing that B_{12} metabolism in algae is associated with the nitrogen-fixing process. To the authors' knowledge, no one has assayed algae for cobamide coenzymes but it seems highly probable that they are present in these organisms.

Occurrence of B_{12} Compounds in Rhizobia and Nodules of Legumes

Early investigations showing that nitrogen-fixing bacteria synthesize vitamin B_{12} compounds were conducted by Burton and Lochhead.[35] Using *L. leichmannii* as the assay organism, they determined the content of B_{12} compounds in *Rhizobium lupini*, *R. meliloti*, *R. trifolii*, *R. leguminosarum*, *R. japonicum*, and *R. phaseoli* grown in pure cultures and concluded that *R. meliloti* was distinguished from the other species examined by its capacity to produce a high amount of the vitamin. No correlation was observed between the capacity to synthesize B_{12} compounds and the effectiveness of the various strains of rhizobia in symbiotic nitrogen fixation. Levin *et al.*[36] used *Ochromonas malhamensis* and *Euglena gracilis* for the determination of B_{12} compounds in pure cultures of rhizobia and in nodules of legumes and have confirmed many of the findings of Burton and Lochhead.[35] In a brief report, they stated that the concentration of B_{12} compounds in root nodules of legumes ranged between 3 and 34 times greater than that in adjacent roots. Additional evidence that the root nodules of leguminous species contain high concentrations of vitamin B_{12} compounds was provided by Okuda and Yamaguchi.[21] By use of *L. leichmannii* and *E. coli* (strain 113–3), they assayed various parts of peas, broad beans, red clover, white clover, alfalfa, and kidney beans and found that the ranges in concentrations of B_{12} compounds (expressed as μg. per 100 gm. of dry materials) were: 0.01 to 0.4 for aerial parts; 0.02 to 3.7 for roots; and 26 to 215 for root nodules. From these observations, it is obvious that rhizobia synthesize vitamin B_{12} compounds and

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that the root nodules of leguminous species contain appreciable quantities of substances with B_{12} activity in microbiological assays.

Cobalt Requirements of Symbiotically Grown Higher Plants

In 1958, experiments were initiated by Evans and associates to determine whether soybean plants (*Glycine max*) grown in symbiosis with *R. japonicum* required cobalt for growth and nitrogen fixation. The first positive evidence from these experiments was reported[37] in 1959. During the same period, Reisenauer[38] grew alfalfa (*Medicago sativa*) in symbiosis with *R. meliloti* and presented evidence that cobalt was necessary for growth and nitrogen fixation. In order to demonstrate a definite cobalt requirement for soybean plants grown in symbiosis with *R. japonicum*, it was absolutely essential to remove cobalt impurities from water and reagent salts. In the experiments with soybean plants,[37,39,40] cobalt contamination was greatly reduced by passing glass distilled water through a column containing ion exchange resins and by extraction of macronutrient salts with 1-nitroso-2-naphthol. Purified water and salt solutions were stored in polyethylene rather than glass vessels. By use of the purified nutrient solution and a special cultural technique which involved the removal of successive harvests of axillary shoots to reduce the cobalt originally present in seeds, a definite cobalt requirement was established for growth of soybean plants in symbiosis with *R. japonicum* (FIGURE 1

FIGURE 1. The effect of cobalt on the growth of soybean plants in symbiosis with *Rhizobium japonicum*. Both cultures of plants were provided with a complete nutrient solution with the exception that nitrogen compounds were omitted and the cobalt concentration in the nutrient was as indicated. The experimental methods have been described by Ahmed and Evans.[39,40]

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The addition of 0.1 ppb* or 1 ppb cobalt to culture solutions resulted in a 12-fold increase in the dry weight of shoots and more than a 22-fold increase in the total nitrogen content of the final harvest of axillary shoots. Without cobalt, 25 per cent of the plants died and those that survived produced an average of only two seeds per plant. In contrast, plants supplied with either 0.1 or 1 ppb cobalt grew vigorously and produced an average of 64 seeds per plant. The addition of twelve elements (listed in FIGURE 2) presumed to have been removed from salts by the purification procedure and not established as essential for higher plants failed to cure the deficient plants. In contrast, the addition of traces of cobalt rapidly alleviated the symptoms. In other experiments, soybean plants were cultured in the absence of symbiotic nitrogen-fixing bacteria and were supplied with a highly purified nutrient solution containing combined nitrogen in nitrate and ammonium salts. Under these conditions no response to cobalt addition was observed.

In California, Delwiche, Johnson, and Reisenauer[41] conducted experiments in which 0.1 micromole of cobalt sulfate was added to each of a series of cultures of alfalfa (*Medicago sativa*) grown in symbiosis with *Rhizobium meliloti*. The addition of this trace element to highly purified nutrient solutions resulted in an increase in the dry weight of plants of more than 100 per cent as compared to controls without added cobalt. The total nitrogen content of tops of plants that received cobalt was approximately nine times greater than that of comparable samples of plants grown without the element. Alfalfa plants behaved like soybeans not responding to cobalt addition when calcium nitrate was supplied in the nutrient medium. By use of N^{15} as a tracer, Delwiche and coworkers[41] determined the nitrogen-fixing capacity of root nodules from cobalt deficient alfalfa. Without added cobalt salts, nodules from deficient plants fixed very little nitrogen, but a small increase in isotope enrichment of nodular material was noted from the addition of 2×10^{-8} *M* cobalt ion to the incubation mixture. They suggested that cobalt was directly involved in the nitrogen-fixing process but, in the opinion of the authors of this paper, the evidence was not conclusive. Presumably cobalt is specifically required for growth of alfalfa in symbiotic association with *R. meliloti*.

That the requirement of cobalt for symbiotically grown leguminous species is not limited to soybean and alfalfa plants was shown by Hallsworth and coworkers[42] in England. In their experiments, the addition of cobalt to cultures of inoculated subterranean clover (*Trifolium subterranean*) increased both the growth and the nitrogen content of the plant material. In a recent publication, Powrie[43] has reported that the addition of cobalt sulfate at the rate of eight ounces per acre to a grey siliceous sandy soil in Australia resulted in a marked increase in the dry weight and nitrogen content of subterranean clover. Certain soils, therefore, not only contain insufficient cobalt to meet the needs of animals but also provide

* The abbreviation ppb refers to parts in 10^9 parts. One ppb is equivalent to 1 μg. per liter.

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an inadequate amount of the element for maximum yields of inoculated legumes grown in symbiosis with rhizobia.

Nonleguminous symbionts also require cobalt for growth and nitrogen fixation. Recently Bond and Hewitt[44] prepared highly purified mineral salts by use of an 8-hydroxyquinoline coprecipitation procedure and have used these for the culture of inoculated alder (*Alnus glutinosa*) and *Casuarina cunninghamiana* in a solution lacking combined nitrogen. The addition of cobalt to nutrient solutions at the rate of 10 ppb resulted in a marked increase in both the dry weight and nitrogen content of alder plants as compared to controls. *Casuarina* exhibited similar responses. It seems apparent, therefore, that cobalt is required for nitrogen fixation and growth of both leguminous and nonleguminous symbionts.

Cobalt Requirement for Nitrogen-Fixing Bacteria

Since it was established that cobalt was an essential element of leguminous species grown in symbiosis with nitrogen-fixing bacteria and apparently was not needed for the growth of the legumes supplied with adequate fixed nitrogen, the question of whether cobalt was an essential micronutrient element for growth of nitrogen-fixing bacteria in pure culture demanded an answer. Positive evidence that *Rhizobium* species required cobalt was provided in 1960.[45,46] In these experiments, the major inorganic components and the carbon source of the nutrient medium were purified by an adaptation of the procedure previously used to study cobalt requirements of soybean plants. Micronutrient elements, excepting iron, were supplied as salts prepared from spectrographic standards or as recrystallized reagent-grade salts. Cobalt was separated from iron by extracting a solution of ferric chloride in 6 N HCl with ethyl ether leaving the cobalt in the aqueous phase. The purified medium was used for the culture of *Rhizobium* species. The addition of 0.5 ppb cobalt to the basal medium prepared from the purified materials resulted in approximately a nine-fold increase in nitrogen content of the cells of *R. japonicum*. Also *R. meliloti*, *R. leguminosarum*, *R. trifolii*, and *R. phaseoli* strikingly responded to an addition of cobalt to the purified medium. The specificity of cobalt as a micronutrient of *R. meliloti* is illustrated in FIGURE 2. The addition to the culture medium of cobalt at a concentration of either 1.0 (flask 1, FIGURE 2) or 0.1 ppb (flask 2, FIGURE 2) resulted in luxuriant growth of bacteria. The addition to the basic medium of 0.1 ppb cobalt (flask 3, FIGURE 2) together with a combination of V, Ni, Ga, Al, Ru, W, Pd, Ag, Sn, Bi, U, and Cr each at a concentration of 0.1 ppb also resulted in excellent growth. The basal medium with the twelve additional elements and without cobalt (flask 4, FIGURE 2) or the basal medium lacking cobalt and the additional elements (flask 5, FIGURE 2) produced no measurable growth of the organism. Since the basal medium contained all the known essential elements including K, P, Mg, S, Ca, N, Fe, Na, Cl, Zn, Mo, B, Mn, and Cu, this experiment proved that cobalt is essential and is highly specific for *R. meliloti*. It represents the first direct demonstration of the essentiality of cobalt for bacteria.

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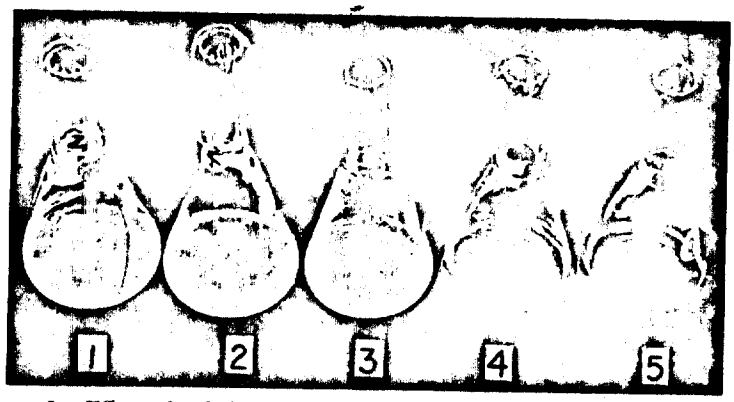

FIGURE 2. Effect of cobalt and 12 other elements on the growth of *Rhizobium meliloti*. The cultures shown in this photograph received the following additions to the basal culture medium: 1, 1.0 ppb Co; 2, 0.1 ppb Co; 3, 12 additional elements (V, Ni, Ga, Al, Ru, W, Pb, Ag, Sn, Bi, U, and Cr) each at a concentration of 0.1 ppb and 0.1 ppb Co; 4, the 12 additional elements previously listed each at a concentration of 0.1 ppb; and 5, no addition. This photograph was supplied through the courtesy of Richie Lowe of our laboratory. The experimental procedure used was described by Lowe and Evans.[46]

Of the various species of *Rhizobium* tested, *R. meliloti* requires the greatest amount of cobalt in the medium. The effect of a graded series of cobalt additions on the growth of *R. meliloti* in a medium containing nitrate is illustrated in FIGURE 3. In the absence of added cobalt, only a

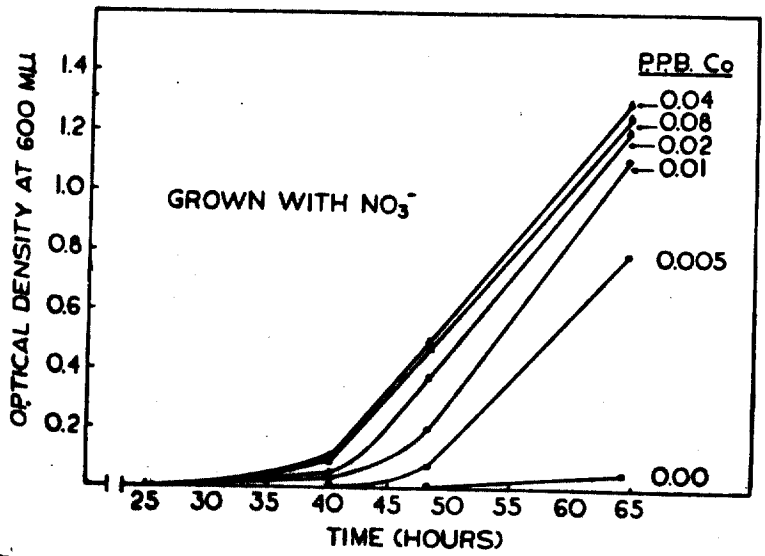

FIGURE 3. Effect of cobalt concentration on the growth of *R. meliloti* in a medium containing nitrate nitrogen. The experimental procedure was essentially the same as that described by Kliewer and Evans.[49]

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trace of growth was shown after 65 hours. During growth periods greater than 65 hours (data not presented), the turbidity of control cultures gradually increased and this was attributed to a slow release of traces of cobalt from walls of glass culture vessels. As illustrated by other curves (FIGURE 3) 0.005 ppb cobalt resulted in a marked increase in growth of *R. meliloti*. At the end of a 65 hour growth period the amounts of growth obtained from 0.02, 0.08, and 0.04 ppb cobalt were approximately the same. Another experiment (FIGURE 4) was conducted in which

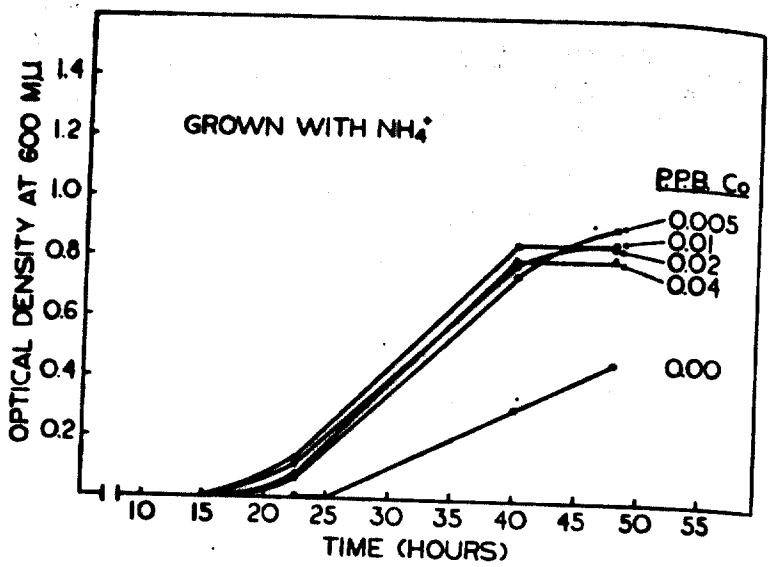

FIGURE 4. Effect of cobalt concentration on the growth of *R. meliloti* in a medium containing ammonium nitrogen. The experimental procedure was essentially the same as that described by Kliewer and Evans.[49]

the effect of cobalt concentrations was determined on the growth of *R. meliloti* in a medium containing ammonium nitrogen. No absolute requirement for cobalt was demonstrated in this case, but a trace of the element obviously was stimulatory. Cultures containing cobalt ranging in concentrations from 0.005 to 0.04 ppb of the element produced maximum growth. From these experiments it appears that the cobalt requirement for *R. meliloti* is greater when the organism utilizes nitrates than when ammonium compounds are utilized. Since ammonium ions are known to form coordination complexes with metals and increase the acidity of culture media, the possibility must be considered that the apparent differential cobalt requirement for the utilization of the two forms of nitrogen resulted from indirect effects.

Nicholas *et al.*[47] have confirmed the observation that *R. japonicum* required cobalt and has noted that the nitrate reductase activity of deficient cells was considerably less than that of cells supplied with a trace

of cobalt. In his experiments, radioactive cobalt failed to accumulate in purified fractions of nitrate reductase prepared from *R. japonicum* cells grown in a culture medium containing the isotope. Nicholas *et al.*[47] concluded that the effect of cobalt on the nitrate reductase activity of this organism may be indirect. In a continuation of these studies, Nicholas *et al.*[48] demonstrated a requirement of cobalt or of vitamin B_{12} for growth of *Azotobacter vinelandii* and claimed that the element was needed for the adaptive formation of nitrate reductase in this bacterium. A correlation was shown between the location of nitrate reductase activity and hydroxocobalamin (labeled with Co^{58}) in ribosomal particles from the bacteria. The data presented by Nicholas *et al.*,[48] and that of Kliewer and Evans[49] (FIGURE 7) are consistent with the conclusion that *A. vinelandii* required a greater concentration of cobalt for the fixation of atmospheric nitrogen than for the utilization of ammonium compounds. Again the possibility was not ruled out that the quantitative differences in response to cobalt when the organism was grown on different forms of nitrogen were caused by indirect effects.

Identification of Cobamide Coenzyme in Symbionts and Nitrogen-Fixing Bacteria

Since vitamin B_{12} compounds were detected in nodules of legumes and in pure cultures of nitrogen-fixing bacteria by microbiological assay procedures, it was of obvious interest to determine whether these organisms contained one or more of the cobamide coenzymes. By use of the glutamate isomerase assay of Barker *et al.*,[51] Kliewer and Evans[49,50] measured the cobamide coenzyme contents of the nodules of nitrogen-fixing symbionts and of cells from pure cultures of rhizobia. A more complete survey (TABLE 1) of the quantities of cobamide coenzyme in nitrogen-fixing organisms reveals that the coenzyme is consistently found in the root-nodules of both leguminous and nonleguminous symbionts. The coenzyme was not detected, however, in the leaves, stems, or roots of inoculated leguminous species. Pure cultures of five *Rhizobium* species, two species of free-living nitrogen-fixing bacteria and two cultures of unidentified actinomycetes from nodules of non-leguminous nitrogen-fixing symbionts contained detectable amounts of coenzyme. Initially it was concluded[50] that some of the coenzyme was free in the nodular mass surrounding the bacteria in root nodules of leguminous species. Further investigation has shown that freezing the nodules released the coenzyme from the bacterial cells. None is detected in nodular material surrounding the bacteria when fresh rather than frozen nodules are assayed.

By use of the extraction procedure and the ion exchange chromatography methods developed by Barker *et al.*,[51] a relatively pure sample of 5,6-dimethylbenzimidazolyl cobamide coenzyme was isolated from pure cultures of *R. meliloti* (Kliewer and Evans).[52] The absorption spectrum and the chromatographic characteristics of the coenzyme isolated from *R. meliloti* were essentially identical with those of crystalline 5,6-dimethyl-

TABLE 1

COBAMIDE COENZYME CONTENT OF NITROGEN-FIXING SYMBIONTS AND MICROORGANISMS*

Species	Part analyzed	No. of samples	Cobamide coenzyme (mμmoles/gm. fresh tissue)†
Medicago sativa	nodules	3	45–64
Glycine max	nodules	8	30–40
Glycine max (nodulated)	roots	2	0
Glycine max (nodulated)	stems	2	0
Glycine max (nodulated)	leaves	2	0
Pisum sativum	nodules	4	36–47
Pisum sativum (nodulated)	roots	2	0
Pisum sativum (nodulated)	stems	2	0
Pisum sativum (nodulated)	leaves	2	0
Trifolium pratense	nodules	2	40
Phaseolus vulgaris	nodules	2	18
Alnus oregona	nodules	5	24–62
Ceanothus velutinus	nodules	2	5–18
Rhizobium meliloti	cells	4	26–30
Rhizobium phaseoli	cells	2	23–25
Rhizobium japonicum	cells	2	15–21
Rhizobium trifolii	cells	2	7–8
Rhizobium leguminosarum	cells	2	7–9
Azotobacter vinelandii	cells	2	3–11
Clostridium pasteurianum	cells	1	4
Unidentified bacteria from nodules of *Ceanothus velutinus*‡	cells	1	2–3
Unidentified actinomycete from nodules of *Ceanothus velutinus*‡	cells	1	1–3

* A portion of the results in this table were reported in a previous publication[50] and are included in this paper by permission of *Nature*.

† Unless otherwise indicated, assays for cobamide coenzyme were conducted by the glutamate isomerase assay method of Barker *et al.*,[51] and results are expressed as amounts of 5,6-dimethylbenzimidazolylcobamide coenzyme.

‡ The organisms were isolated from nodules of *Ceanothus velutinus* by Arthur Wollum, Oregon State University, Corvallis, Oregon.

benzimidazolyl-cobamidecoenzyme (FIGURE 5). The same procedures were used to isolate a cobamide coenzyme from soybean nodules and this compound also has been identified as the 5,6-dimethylbenzimidazolyl-cobamide coenzyme (FIGURES 5 and 6). During the isolation procedures, components were observed in both *R. meliloti* and soybean nodule extracts that were presumed to be derivatives of the B_{12} coenzymes, but none of these were identified and none exhibited activity in the glutamate isomerase assay. It seems probable that a major cobamide coenzyme component of *R. meliloti* and soybean nodules is the 5,6-dimethylbenzimidazolylcobamide compound. According to a statement by Nicholas *et al.*,[48] this coenzyme also has been identified in *Azotobacter vinelandii*.

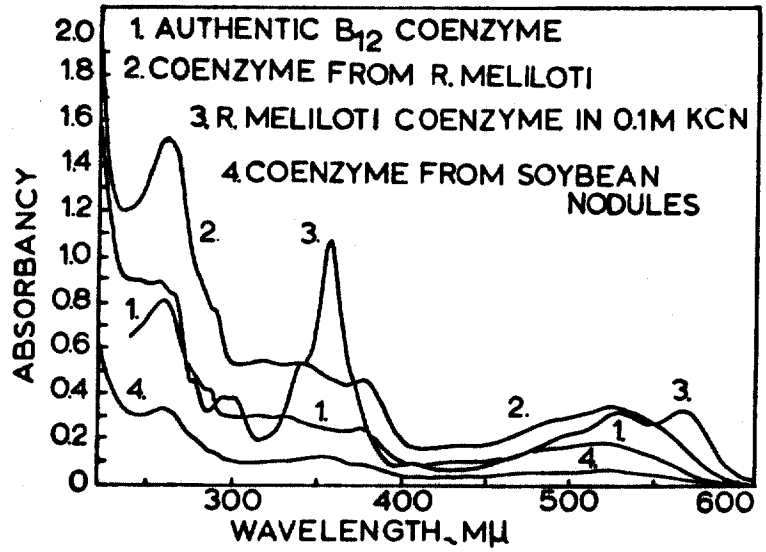

FIGURE 5. Absorption spectra of (1) authentic DBC coenzyme, (2) coenzyme from *R. meliloti* in 0.01 *M* potassium phosphate buffer at *p*H 6.8, (3) *R. meliloti* coenzyme in 0.1 *M* KCN and (4) coenzyme from soybean nodules in 0.01 M potassium phosphate, *p*H 6.8. The spectra labelled (1) and (2) were published previously[52] and included here by permission of *Plant Physiology*.

Vitamin B_{12} Compounds in Nitrogen-Fixing Organisms in Relation to the Cobalt Supply

Several workers[22,53] have noted that the addition of cobalt to nutrient media used for the culture of microorganisms increased the synthesis of vitamin B_{12} compounds. In most of these investigations, the media utilized was not purified and, therefore, the effect of cobalt deficiency on the synthesis of the B_{12} compounds was not determined. In the initial investigation of the cobalt requirement of soybean plants grown in symbiosis with *R. japonicum*, root nodules were collected from plants receiving different cobalt treatments and were assayed for B_{12} compounds by use of *Ochromonas malhamensis*. The striking effect of the cobalt additions to purified nutrient solutions on the amount of vitamin B_{12} compounds found in nodules is illustrated in TABLE 2. In this and other experiments, little vitamin B_{12} compounds could be detected in nodules from plants grown in the absence of added cobalt. The addition of 1 ppb cobalt to the culture solution resulted in a marked increase in the amount of B_{12} compounds in nodules. The nutrient solution containing the highest cobalt concentration (50 ppb) resulted in a luxury synthesis of the vitamin. This concentration of cobalt, however, caused no greater growth response than was obtained with 1 ppb of the element in the medium.

In another experiment, the effect of a series of increasing concentrations of cobalt in a purified culture medium on the growth and the cobam-

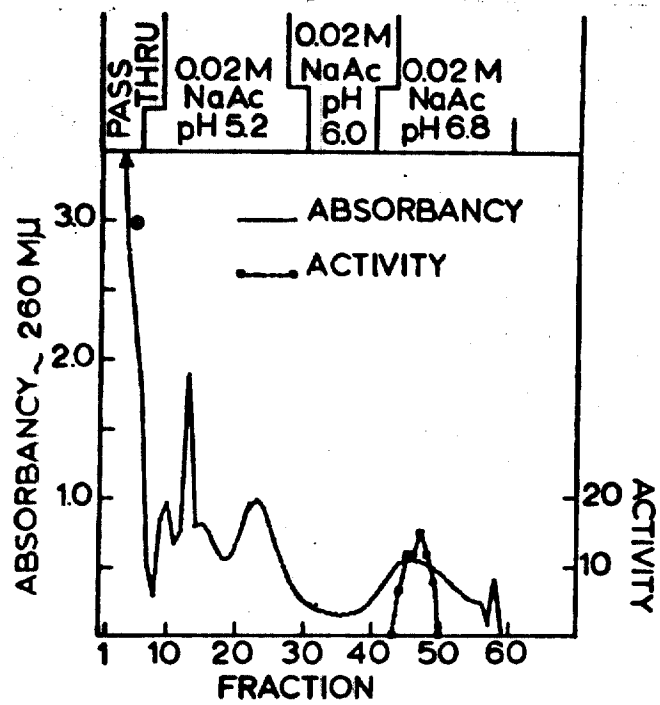

FIGURE 6. Separation of cobamide coenzyme from soybean nodules on a Dowex 50W column.* The column was eluted with the sodium acetate buffers indicated. A unit of activity is the amount of coenzyme that causes an absorbancy change of 0.01 per minute in the glutamate isomerase assay described by Barker *et al.*[51] The spectrum of those fractions showing activity in the glutamate isomerase reaction is presented in FIGURE 5.

TABLE 2

EFFECT OF COBALT IN CULTURE SOLUTIONS ON THE CONTENT OF VITAMIN B_{12} COMPOUNDS IN ROOTS NODULES FROM SOYBEAN PLANTS*
(FROM AHMED AND EVANS[39])†

Addition to standard nutrient solution*	Vitamin B_{12}‡ mμgm./gm. fresh wt.
None	0.1
1 ppb Co as vitamin B_{12}	7.8
1 ppb Co as $CoCl_2$	0.7
50 ppb Co as $CoCl_2$	519.3

* The purification method for this experiment and the composition of the standard nutrient solution are described in detail by Ahmed and Evans.[39,40]

† Data in this table are reproduced by permission of *Soil Science*.

‡ All values reported are means of determinations on four replicate samples. B_{12} compounds were determined by use of *Ochromonas malhamensis* as the assay organism.

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ide coenzyme content of pure cultures of *Rhizobium meliloti* was investigated (TABLE 5). The addition of cobalt at a concentration of either 0.01 or 1 ppb resulted in a striking increase in growth of the bacteria but higher concentrations (1000 ppb) resulted in growth depression when compared with the treatment containing the optimum concentration. The cobamide coenzyme content (TABLE 3) of cells from cultures

TABLE 3

EFFECT OF COBALT ON THE GROWTH AND ON THE CONTENTS OF NITROGEN AND B_{12} COENZYME IN RHIZOBIUM MELILOTI* (FROM KLIEWER AND EVANS[49])‡

Co in medium (ppb)	Fresh wt. of cells* (mg./culture)	Total N* (mg./culture)	B_{12} coenzyme in fresh cells* (mμmoles/gm.)‡
0.00	328	5.0	<1.0
0.01	1278	18.8	<1.0
1.00	1300	20.0	10.7
100.00	1279	24.6	27.8
1000.00	680	13.5	35.9
L.S.D. (5%)	660	12.5	11.1

* All values reported are means of determinations on three replicate cultures.
† Data in this table published with the permission of *Plant Physiology*.
‡ Coenzyme contents are expressed as the 5,6-dimethylbenzimidazolylcobamide coenzyme.

lacking added cobalt or supplied with 0.01 ppb of the element was too low for measurement. Increasing the cobalt in the medium in increments up to 1000 ppb resulted in a steady increase in the cobamide coenzyme content of the cells. These results show that the organism synthesizes a luxury amount of cobamide coenzyme when excessive cobalt is available.

It was desired to determine whether the concentration of cobalt required for growth and for fixation of atmospheric nitrogen by a representative microorganism was different from that required for the utilization of ammonium compounds. For this comparison the use of a free-living nitrogen-fixing organism was necessary. The effect of cobalt additions to purified media on the growth of *Azotobacter vinelandii* with either atmospheric nitrogen or ammonium nitrogen is illustrated in FIGURE 7. It is apparent that the addition of cobalt to cultures of *A. vinelandii* grown with atmospheric nitrogen strikingly stimulated growth, the maximum resulting from concentrations ranging between 0.01 and 1 ppb in the medium. A concentration of 1000 ppb was excessive. A curve exhibiting a similar maximum was obtained when ammonium nitrogen was used, but in this case the relative response from cobalt was much less than that obtained when atmospheric nitrogen was the sole nitrogen source. One may conclude that *A. vinelandii* required a greater amount of cobalt for growth when utilizing atmospheric nitrogen than when utilizing ammonium compounds. In the same experiment (FIGURE 5), the content of

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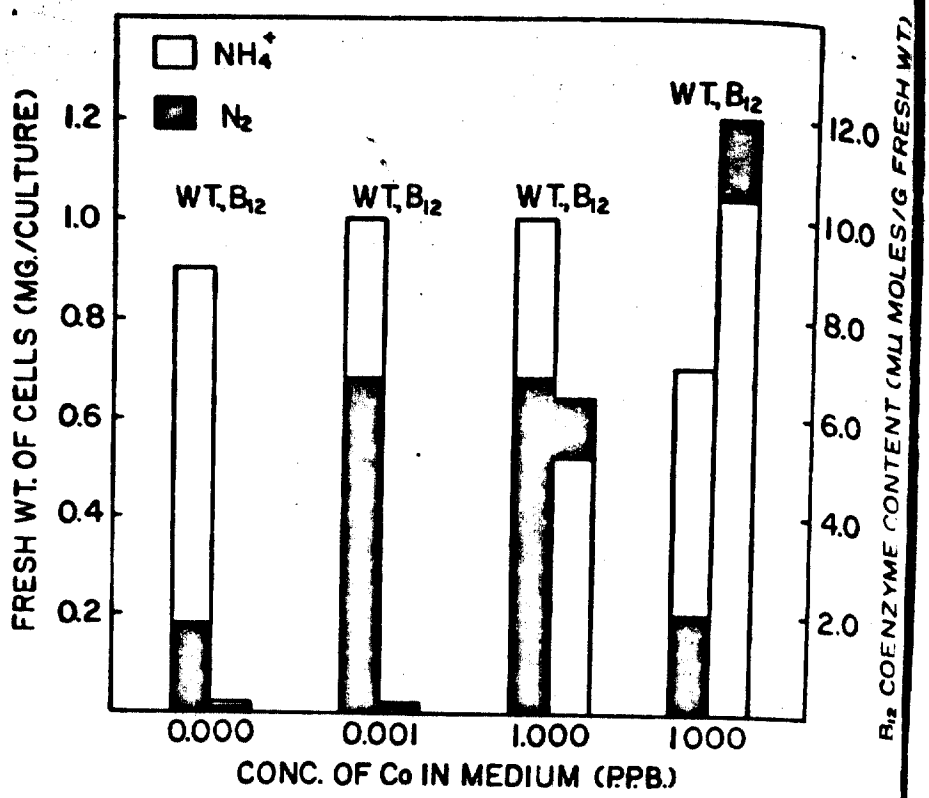

FIGURE 7. Effect of the concentration of added cobalt and the source of nitrogen (NH_4^+ or N_2) on the growth and the cobamide coenzyme content of *Azotobacter vinelandii*. Growth is indicated in the left bar at each Co concentration as "Wt." and B_{12} coenzyme content in the right bar at each Co concentration as "B_{12}." The glutamate isomerase assay[51] was used to determine the B_{12} coenzyme content of cells. Crystalline 5,6 dimethylbenzimidozolylcobamide coenzyme was used as a standard. Cultures were harvested after a growth period of 24 hours. Methods used in a similar experiment have been described by Kliewer and Evans.[49]

cobamide coenzyme in cells from cultures was markedly influenced by the amount of cobalt in the culture medium but was not appreciably influenced by the source of nitrogen utilized by the organism.

Role of Cobamide Coenzymes in Nitrogen-Fixing Organisms

Very little information is available concerning the role of the cobamide coenzymes in organisms that fix atmospheric nitrogen. In our laboratory, efforts have been made to identify enzymes in nodules of legumes and in pure cultures of *Rhizobium* species that require cobamide coenzymes for activity. Several attempts to detect activities of glutamate

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isomerase[51] and glycerol dehydrase[54] have yielded negative results. On the other hand, methylmalonyl Co A mutase[55] appears to be widely distributed in nodules of symbionts and nitrogen-fixing bacteria.[56] In a series of unpublished experiments, August De Hertogh of our laboratory has demonstrated methylmalonyl Co A mutase activity in cell-free extracts of bacteroids from the nodules of peas, alfalfa, and soybean plants. No enzyme activity was detected in tissues of the higher plants. Also the enzyme has been detected in extracts prepared from pure cultures of *R. meliloti*, *R. trifolii*, *R. phaseoli*, *R. leguminosarum*, and *R. japonicum*. Subjection of an extract of *R. meliloti* to visible light resulted in inactivation of methlymalonyl Co A mutase and the activity was restored by the addition of the 5,6-dimethylbenzimidazolyl cobamide coenzyme. With this preparation, the adenylcobamide coenzyme failed to reverse the inactivation caused by the light treatment. *Rhizobium* species and bacteroids from legume nodules not only contain methylmalonyl Co A mutase but all specimens tested[57] have shown exceptionally active propionyl Co A carboxylase activities. Also several *Rhizobium* species exhibit an absolute carbon dioxide requirement for growth. Additional research is needed to elucidate the role of methylmalonyl Co A mutase and related enzymes in the metabolism of *Rhizobium* species. Some of these problems are now being investigated in our laboratory.

A series of experiments were conducted to determine whether or not cobamide coenzymes play a role in the pyruvate dependent nitrogen-fixation reactions catalyzed by cell-free extracts of *Clostridium pasteurianum*.[58] Vigorous nitrogen fixation was consistently demonstrated with extracts from this organism. In some experiments, the addition of B_{12} coenzyme to extracts previously subjected to light treatment resulted in increased nitrogen fixation. Response to added B_{12} coenzyme was not consistent in all experiments and requires further confirmation. Obviously further research is essential to elucidate the biochemical role of the cobamide coenzymes in the metabolism of the organisms that fix atmospheric nitrogen. At the present time, insufficient evidence is available to determine whether the roles of B_{12} coenzymes in organisms that fix atmospheric nitrogen is any different from those in organisms that lack nitrogen-fixing capacities. It is believed that the use of the techniques for producing cobalt deficiency in bacteria will continue to be useful in studying the role of the B_{12} coenzymes in the metabolism of nitrogen-fixing organisms.

Summary

Vitamin B_{12} compounds are widely distributed in organisms that fix atmospheric nitrogen. The vitamin has been detected in blue-green algae, nodules of both leguminous and nonleguminous symbionts, *Rhizobium* species, *Azotobacter vinelandii*, and *Clostridium pasteurianum*. Small quantities of vitamin B_{12} compounds have been detected in tissues of higher plants but no compelling evidence is available showing that higher plants *per se* possess the capacity to synthesize the vitamin. It seems

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highly probable that the vitamin B_{12} compounds that have been found in occasional samples of higher plant tissues were derived from microbial origin.

Cobalt is required for the growth of certain blue-green algae, leguminous plants grown in symbiotic association with *Rhizobium* species, *Rhizobium* grown in pure culture, *A. vinelandii*, and *Alnus glutinosa* and *Casurina* grown in symbiotic association with unidentified endophytes. From the limited evidence available, it appears that all nitrogen-fixing organisms that have been thoroughly investigated require cobalt for growth and synthesis B_{12} compounds. On the other hand, no conclusive experimental evidence is available showing a cobalt requirement for higher plants grown in the absence of symbiotic microorganisms.

Cobamide coenzymes have been detected in appreciable quantity in *Rhizobium* species, root nodules of both leguminous and nonleguminous symbionts, *A. vinelandii* and *Clostridium pasteurianum*. The quantity of cobamide coenzyme synthesized by *Rhizobium* species and *A. vinelandii* is strikingly influenced by the cobalt supply in culture media. The particular B_{12} coenzyme present in the nodules of soybean plants and pure cultures of *Rhizobium meliloti* and *Azotobacter vinelandii* has been identified as the 5,6-dimethylbenzimidazolylcobamide compound.

A. vinelandii, grown under conditions where fixation of atmospheric nitrogen is obligatory, requires a greater concentration of cobalt than is needed for utilization of ammonium compounds. The possibility that the difference in cobalt requirement for use of various nitrogen sources is caused by some indirect effect has not been ruled out.

Cells from pure cultures of *Rhizobium* species and from nodules of leguminous plants contain appreciable amounts of methylmalonyl Co A mutase. The enzyme in extracts of *R. meliloti* requires the 5,6-dimethylbenzimidazolylcobamide coenzyme for activity. No other enzymes requiring the cobamide coenzymes have been detected in extracts of nitrogen-fixing organisms. Further investigation is necessary to elucidate the role of cobamide coenzymes in the metabolism of nitrogen-fixing organisms and to determine whether or not the roles of these coenzymes in nitrogen-fixing organisms is any different from roles established in organisms lacking nitrogen-fixing capacities.

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POULTRY SCI 34, 922-7 (1955)

The Vitamin B_{12} Content of Fresh and Stored Shell Eggs[1]

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(Received for publication December 10, 1954)

THE vitamin B_{12} content of fresh and stored shell eggs has been determined as part of a study of changes in composition occurring when shell eggs are kept in cold storage. Losses of nutrients during holding and storage of shell eggs are of importance, because large numbers of eggs are stored for extended times and because fresh eggs are usually several days old before they are eaten.

According to previous work from this laboratory, eggs stored for twelve months lost 18 percent of the niacin, 51 percent of the vitamin B_6, 14 percent of the riboflavin, 8 percent of the pantothenic acid, and 27 percent of the folic acid (Evans *et al.*, 1951b, c, 1952a, b, 1953a) but did not lose choline nor biotin (Evans *et al.*, 1951a, 1953b). Data are herein presented which show a loss of vitamin B_{12} from shell eggs that had been stored for twelve months.

EXPERIMENTAL

Two studies were conducted. Eggs for the first were obtained in March 1951 and those for the second in March 1953. The eggs used in both studies were produced by S.C. White Leghorn hens housed in individual laying cages and fed a ration of constant composition. The ration contained ground corn 34.5 percent, ground oats 20.0 percent, wheat bran 15.0 percent, flour middlings 10.0 percent, dehydrated alfalfa 3.0 percent, meat

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scraps 3.0 percent, dried milk 2.0 percent, fish meal 2.5 percent, soybean oil meal 2.5 percent, ground oyster shell flour 5.0 percent, steamed bone meal 1.5 percent, salt 0.6 percent, and fish oil (400D and 3,000A) 0.4 percent. Eggs were gathered daily, marked with hen number and date, and placed in cold storage at 0°C. or assayed for vitamin B_{12}. Eggs from ten hens were used in each experiment. Three eggs from each hen were immediately assayed for vitamin B_{12}, and twelve more were stored in a carton holding one dozen eggs. The ten cartons were kept in a fiber egg case in a walk-in refrigerator set at 0°C. Three eggs from each hen were assayed for vitamin B_{12} after 3, 6, and 12 months of storage in the first experiment and after 3, 7, and 12 months of storage in the second.

The three eggs from each hen were hard boiled by keeping them in boiling water for 20 minutes, cooled under running water, broken out, and yolks and whites separated and weighed. Yolks and whites of the three eggs from each hen were separately composited and duplicate 10.0 gram samples used. Each sample in the first experiment was hydrolyzed by incubation under toluene at 40° C. for 48 hours with 50 ml. H_2O containing 1.5 grams K_2HPO_4 and 50 milligrams of trypsin and adjusted to pH 8.0 with phenolphthalein as an indicator. The second experiment egg samples were autoclaved at 15 pounds pressure for 15 minutes with a solution of 25 ml. of 0.1 M phosphate-citrate buffer of pH 4.5 containing 25 mg. of sodium bisulfite.

The digests were neutralized, filtered, and suitable dilutions made for assay. Vitamin B_{12} was determined using *Lactobacillus leichmannii* as the assay organism, the assay procedure of Skeggs *et al.* (1950), and a double strength medium containing in each 100 ml., casein hydrolysate 2.0 gm., glucose 2.0 gm., sodium acetate 0.6 gm., Tween 80 0.2 gm., thiomalic acid 100 mg., tryptophan 40 mg., cystine 20 mg., adenine 2 mg., guanine 2 mg., uracil 2 mg., xanthine 2 mg., salts A 1 ml., salts B 1 ml., riboflavin 200 mcg., thiamine 200 mcg., para-aminobenzoic acid 20 mcg., calcium pantothenate 200 mcg., pyridoxine 400 mcg., niacin 200 mcg., biotin 1 mcg., folic acid 100 mcg., pyridoxal 250 mcg., and pyridoxamine 250 mcg.

Digestion with trypsin was the accepted procedure for liberation of vitamin B_{12} from biological materials when the first experiment was started. A method study was made at the end of the first experiment, because of putrefaction of egg samples from bacterial growth when 12-month old eggs were digested with trypsin even when the digestion was done under toluene. The methods studied were based on those used by Denton and Kellogg (1953) for eggs and the trypsin digestion procedure.

Ten-gram samples of egg yolk or white were hydrolyzed by incubating with trypsin (two different samples), by autoclaving with 25 ml. of 0.1 M phosphate-citrate buffer of pH 4.5, by autoclaving with 25 ml. of the buffer and 25 mg. of sodium bisulfite, or by autoclaving with 25 ml. of the buffer and 10 mg. of sodium cyanide.

RESULTS

Average values for the total vitamin B_{12} and millimicrograms vitamin B_{12} per gram of egg yolk in fresh, 3 month old, and 6 month old eggs from the first experiment are given in Table 1. No vitamin B_{12} was found in egg whites after trypsin digestion. Putrefaction occurred in the 12-month old eggs during digestion with trypsin even though the digestion was done under toluene, and very high

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TABLE 1.—*Changes in the vitamin B_{12} content of egg yolks during storage of shell eggs (Expt. 1)*

Hen No.	Fresh eggs, millimicrograms	3 month old eggs, millimicrograms	6 month old eggs, millimicrograms
6605	151	248	188
6607	150	197	139
6608	174	225	192
6612	135	165	139
6613	154	191	158
6620	143	158	139
6621	165	181	158
6625	123	178	126
6626	158	301	212
6633	90	158	141
Ave.	144	200	159
	millimicrograms/gm.	millimicrograms/gm.	millimicrograms/gm.
Ave. millimicrograms vitamin B_{12} per gm. of yolk	8.66	11.16	8.88

vitamin B_{12} values were obtained. A new sample of trypsin was used, and the contamination probably came from it, but no additional eggs were available from this study to repeat the digestion using the old trypsin sample. Fresh egg yolk samples were digested with the old and the new trypsins. Putrefaction and high vitamin B_{12} assay values were obtained in egg samples digested with the new trypsin but not in those digested with the old one (Table 2).

Less vitamin B_{12} was liberated from egg yolk or egg white by incubating with trypsin (except for trypsin sample B where putrefaction occurred) than by autoclaving with either sodium cyanide or sodium bisulfite in buffer solution (Table 2). Poor liberation of vitamin B_{12} was obtained by autoclaving with the buffer alone. Sodium bisulfite and sodium cyanide were further compared in two additional experiments as additives to the buffer when the egg samples were autoclaved. Yolks and whites of raw and boiled eggs were used (Table 2). The method decided upon because of results obtained from this study, was to autoclave the cooked egg yolk or white with a buffer containing sodium bisulfite. This procedure gave more consistent results than any of the others and was easier and safer to use. The cooked whites assayed higher in vitamin B_{12} than the raw ones, but the raw yolks appeared to contain slightly more vitamin B_{12} than the cooked ones.

A small amount of vitamin B_{12} was present in the whites of eggs used in the second experiment (Table 3). Fresh egg white contained 11 to 39 (ave. 25) millimicrograms of vitamin B_{12} per egg or 0.27 to 1.00 (ave. 0.63) millimicrograms per gram of white. No change in content of vitamin B_{12} in egg white occurred during 12 months of cold storage. The vitamin B_{12} concentration in the whites increased during the last five months of storage from

TABLE 2.—*Comparison of methods for digesting egg samples preliminary to assay* (millimicrograms per gm.)

	Digestion method	Cooked eggs		Raw eggs	
		White	Yolk	White	Yolk
Experiment 1	Trypsin A	.00	6.80		
	Trypsin B	.00	(19.40)*		
	Autoclaved	.01	4.40		
	Auto. cyanide	.07	11.32		
	Auto. bisulfite	.12	10.36		
Experiment 2	Auto. cyanide	.30	11.12	.03	17.43
	Auto. bisulfite	.40	12.52	.05	15.27
Experiment 3	Auto. cyanide	.17	10.52	.10	11.37
	Auto. bisulfite	.14	8.90	.10	9.06

* Putrefaction occurred in this sample during digestion.

TABLE 3.—*Changes in the vitamin B_{12} content of egg whites during storage of shell eggs (Expt. 2)*

Hen No.	Fresh eggs, millimicrograms	3 month old eggs, millimicrograms	7 month old eggs, millimicrograms	12 month old eggs, millimicrograms
7203	34	28	35	25
7206	11	10	6	7
7207	18	17	17	18
7209	29	24	26	22
7213	18	19	22	24
7214	22	30	27	29
7215	39	35	34	33
7217	23	16	26	24
7218	25	17	18	18
7219	28	27	22	31
Ave.	25	22	21	23
	millimicrograms/gm.	millimicrograms/gm.	millimicrograms/gm.	millimicrograms/gm.
Ave. millimicrograms vitamin B_{12} per gm. of white	0.63	0.63	0.63	0.77

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0.63 millimicrograms per gram in 7-month old eggs of 0.77 millimicrograms per gram in 12-month old eggs because of loss of water from the white.

There was quite a difference in the amount of vitamin B_{12} that individual hens put in their eggs (Table 4). For example hen No. 7206 put an average of 112 millimicrograms of vitamin B_{12} in each yolk (5.82 millimicrograms per gm.) and hen No. 7212 put 678 millimicrograms in a yolk (32.64 millimicrograms per gm.). The other hens deposited amounts of vitamin B_{12} in their eggs in between what these two did.

On the average 11.9 percent of the vitamin B_{12} was lost from yolks of shell eggs (second experiment) during the first three months of cold storage (Table 4). There was no change during the next four months, but by the end of twelve months 34.3 percent of the original vitamin B_{12} had been lost. The concentration of vitamin B_{12} in egg yolks also decreased from 18.53 millimicrograms per gram to 11.35 millimicrograms per gram (Table 4). Statistical analysis gave a t value of -2.38 between storage time and millimicrograms of vitamin B_{12} per gram of yolk and one of -2.03 between storage time and millimicrograms of vitamin B_{12} per egg yolk. A t value of 2.00 was required for significance at the 5 percent level.

TABLE 4.—*Changes in the vitamin B_{12} content of egg yolks during storage of shell eggs (Expt. 2)*

Hen No.	Fresh eggs, millimicrograms	3 month old eggs, millimicrograms	7 month old eggs, millimicrograms	12 month old eggs, millimicrograms
7203	246	235	202	166
7206	112	88	105	61
7207	420	328	281	170
7209	451	378	368	246
7213	288	299	272	212
7214	678	525	441	451
7215	576	513	470	434
7217	334	281	424	247
7218	252	234	354	185
7219	266	310	262	179
Ave.	362	319	318	237
	millimicrograms/gm.	millimicrograms/gm.	millimicrograms/gm.	millimicrograms/gm.
Ave. millimicrograms vitamin B_{12} per gm. of yolk	18.53	15.21	15.26	11.35

TABLE 5.—*Changes in the vitamin B_{12} content of whole eggs during storage of shell eggs (Expt. 2)*

Hen No.	Fresh eggs, millimicrograms	3 month old eggs, millimicrograms	7 month old eggs, millimicrograms	12 month old eggs, millimicrograms
7203	280	263	227	191
7206	123	98	111	68
7207	438	345	298	188
7209	480	402	394	268
7213	306	318	294	236
7214	700	555	468	480
7215	615	548	494	487
7217	357	297	450	271
7218	277	251	372	203
7219	294	337	284	210
Ave.	387	341	339	260
	millimicrograms/gm.	millimicrograms/gm.	millimicrograms/gm.	millimicrograms/gm.
Ave. millimicrograms vitamin B_{12} per gm. of egg	6.54	6.07	6.17	5.03

Whole eggs contained little more vitamin B_{12} than egg yolks (387 compared to 362 millimicrograms per egg), but the millimicrograms per gram was much less in the whole egg (6.54) than in the egg yolk (18.53) because of the diluting effect of the white, which contained little vitamin B_{12} (Table 5). Losses of vitamin B_{12} from the whole egg during storage paralleled losses from the yolk. Thirty-two and eights tenths percent, on the average, of the vitamin B_{12} was lost from whole eggs during twelve months of cold storage. This is roughly one-third of the vitamin B_{12} originally present.

DISCUSSION

Egg yolk may be considered a good source of vitamin B_{12}. The 1.85 mcg. of vitamin B_{12} per 100 grams of egg yolk (Table 4) compares well with 1.3–4.5 mcg. per 100 gm. of beef round, 1.7–3.2 mcg. per 100 gm. of leg of lamb, 2.2 mcg. per 100 gm. of lamb shoulder chops, 2.9 mcg. per 100 gm. of beef rib, 1.6 mcg. per 100 gm. of pork tenderloin, and 0.9–2.1 mcg. per 100 gm. of pork ham (Scheid and Schweigert, 1951; Scheid *et al.*, 1952),

or with the 0.32–1.60 mcg. per 100 ml. of milk (Collins *et al.*, 1951), or the 3.0–4.0 mcg. per 100 gm. of chicken leg muscle and 2.4–3.4 mcg. per 100 gm. of chicken breast muscle (Yacowitz *et al.*, 1952b), but is much lower than the 30–99 mcg. per 100 gm. of fresh beef liver (Scheid *et al.*, 1952). The decrease in vitamin B_{12} concentration in egg yolks of from 1.85 to 1.14 mcg. per 100 gm. is of importance from a nutritional standpoint especially where eggs may furnish an appreciable amount of the animal protein in the diet.

The question may be raised as to whether the microbiological assay procedure used measures something besides vitamin B_{12} in eggs. The authors have not studied this problem nor do they know of any such studies, but they believe that all losses during storage are of vitamin B_{12} and not of the alkali stable thymidine if any is present in egg yolks.

Values obtained for the vitamin B_{12} content of whole eggs and for the millimicrograms per gram of egg yolk presented in Tables 4 and 5 agree well with published work of others. Some variation would be expected because of differences in vitamin B_{12} content of diets used and because of differences in amount of vitamin B_{12} put in the egg by different hens receiving the same ration. Halick *et al.* (1953) found that the vitamin B_{12} content of the egg depended upon the vitamin B_{12} content of the hen's diet. Values of 0.27 mcg. of vitamin B_{12} per egg reported by Csonka *et al.* (1954), and the 0.12–0.26 mcg. per egg by Milligan *et al.* (1952) are within the range of 0.12–0.70 mcg. per fresh egg laid by the hens used in the second experiment. The 5.82–32.64 millimicrograms vitamin B_{12} per gm. of egg yolk found in the second experiment eggs agree well with the 30 millimicrograms per gm. found by Lillie *et al.* (1954), the 3.7–67.3 millimicrograms per gm. by Halick *et al.* (1953), the 2.80–15.56 millimicrograms per gm. by Yacowitz *et al.* (1952a), the 5.0–25.0 millimicrograms per gm. by Olcese *et al.* (1950), and the 24.0 millimicrograms per gm. by Denton and Kellogg (1953). Chick feeding assays of Milligan *et al.* (1952) indicated nearly ten times as much vitamin B_{12} in eggs as did their microbiological assays.

Individual hens differed in their ability to place vitamin B_{12} in their eggs. The cause of this difference is not known, but it is probably the result of differences among the hens in their digestion, absorption, or metabolism of dietary vitamin B_{12} or in the mechanism having to do with egg formation. Similar differences have been observed for most of the other vitamins studied, although few showed as large differences as was the case for vitamin B_{12}. Eggs from hen No. 7206 had especially low vitamin B_{12} content. One would suspect some defect in this hen's metabolism. The low vitamin B_{12} content of eggs from hen No. 7206 raises several interesting unanswered questions. Would fertile eggs from this hen hatch, and if they did would the chicks have sufficient reserves of vitamin B_{12} to last them until dietary vitamin B_{12} could meet their needs? Could the vitamin B_{12} content of the eggs be increased by feeding this hen large doses of vitamin B_{12}, or is there a defect in its ability to place this vitamin in the eggs? A study of the metabolism of hens such as this one would be extremely interesting and of value for increasing our knowledge of vitamin B_{12} absorption and metabolism.

There was a 32.8 percent loss of vitamin B_{12} from the second experiment eggs during 12 months of storage but none was indicated during six months in the preliminary experiment. The greatest loss of vitamin B_{12} from the second experiment eggs occurred between the seventh

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and twelfth months of storage, a period for which no data are available for the preliminary experiment.

A statistical t value of -2.38 was obtained between storage time and vitamin B_{12} per gram of yolk and one of -2.03 between storage time and vitamin B_{12} in each egg yolk. A t value of 2.00 was required for significance at the five percent level. A more highly significant statistical t value would be expected, because there was a loss of one-third of the vitamin B_{12} during twelve months. The loss of vitamin B_{12} was not a straight line function. Ten percent initial loss during the first three months was followed by four months with no loss, and the greatest loss occurred during the last five months. The t value applies to a straight line function, and the values obtained for three and seven month old eggs do not fit a straight line connecting the values for fresh and twelve month old eggs. If only fresh and twelve month or seven and twelve month values were used the differences should be statistically very highly significant because eggs from every hen lost vitamin B_{12}.

What is the significance of these data from the viewpoint of human nutrition? The ultimate purpose of producing and storing eggs is for human consumption. As was pointed out earlier, eggs are one of the good sources of vitamin B_{12} in man's diet. Loss of one-third of the vitamin B_{12} could appreciably reduce the vitamin B_{12} intake and might possibly contribute to a vitamin B_{12} deficiency. The vitamin B_{12} requirements of the healthy man are still not well defined. Although fresh eggs are unquestionably to be preferred to stored ones, eggs stored under good conditions for as long as seven months did not lose very much vitamin B_{12}, but longer storage should be avoided if the eggs are to retain their vitamin B_{12}.

SUMMARY

Fresh shell eggs contained on the average 387 millimicrograms of vitamin B_{12} per egg (6.54 millimicrograms per gm.) with 362 millimicrograms (18.53 millimicrograms per gm.) in the yolk and 25 millimicrograms (0.63 millimicrograms per gm.) in the white. No change in the vitamin B_{12} content of the egg whites was observed during 12 months of cold storage, but about one-third of the vitamin B_{12} was lost from the whole egg. Most of this loss occurred during the last five months of storage. Twelve month old eggs contained on the average 260 millimicrograms of vitamin B_{12} per egg (5.03 millimicrograms per gm.) with 237 millimicrograms in the yolk (11.35 millimicrograms per gm.), and 23 millimicrograms in the white (0.77 millimicrograms per gm.).

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Metabolic Patterns in Preadolescent Children

VI. VITAMIN B_{12} INTAKE AND URINARY EXCRETION[1]

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Man is dependent upon animal foods for his chief source of vitamin B_{12}. Dietary deficiency of this vitamin has been reported for human beings consuming vegetable diets exclusively (Wokes et al., '55). But there is a general lack of information on the vitamin B_{12} content of diets made up of ordinary foods.

The study reported here was undertaken to obtain information on the vitamin B_{12} content of controlled diets used in a series of studies on metabolic patterns and utilization of selected nutrients by preadolescent girls. Twelve 7- to 9-year-old girls participated in the 1956 study and 11 other girls of like age in 1958. Average daily urinary excretion of vitamin B_{12} was determined for the 11 girls in 1958 when diets of low-protein content were used.

Information on the weighed portions of foods used in the meals for each of the 6 diets, as well as the physical and biochemical characteristics of each girl, are presented in the Southern Cooperative Series Bulletin no. 64 ('59).

PLAN OF STUDIES

The 1956 study consisted of 14 4-day metabolic periods and the 1958 study of 8 6-day periods. A different menu was used on each day within a metabolic period, the menus being repeated in the same order for each period.

All menus were planned to provide the levels of nutrients recommended in the report of the Food and Nutrition Board ('53) except that protein was higher than the recommended amount of 2 gm per kg body weight for 6 of 12 girls in 1956 and lower for all girls in 1958. In 1958, however, the diets provided a sufficient amount of protein to allow for a nitrogen retention of at least 0.3 gm per child per day (James, '60). Hydrolyzed gelatin was used in some diets in 1956 to furnish a portion of the protein (Southern Coop. Series Bull. no. 64, '59).

MATERIALS AND METHODS

Daily at mealtime, weighed aliquots of one-half portion of every food served were placed in a Pyrex jar and frozen. The daily food composites for one metabolic period were blended in a Waring Blendor until the slurry was homogeneous.

All urine for each girl was collected for 24-hour periods, kept under refrigeration during the collection period and held frozen until pooled for each metabolic period.

All samples of pooled urine for each girl and food slurries for the group of girls were kept frozen until the time of analyses.

Vitamin B_{12} was determined by microbiological assays with *Lactobacillus leichmannii* using the AOAC ('55) method with minor modifications (Lichtenstein et al., '61). To insure that the growth promoting activity measured was derived from vitamin B_{12}, checks were carried out with *Ochromonas malhamensis* using a modification (Lichtenstein et al., '61) of Ford's ('53) method.

RESULTS AND DISCUSSION

In table 1 is presented the protein (N × 6.25) content of each of the 6 diets used

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[1] This study was a phase of the Southern Regional Research Project, Requirements and Utilization of Selected Nutrients by Preadolescent Children, supported in part by funds appropriated to the U. S. Department of Agriculture under the Research and Marketing Act of 1946, and the Hatch Act, as amended. The Human Nutrition Research Division of the Agricultural Research Service was a cooperator in this project.

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In general, very little vitamin B_{12} was excreted in the urine with these low-protein diets. For the first 6-day period, average daily urinary excretion values ranged from 0.04 to 0.22 μg per day indicating a fivefold variation among the subjects. By the third period at least, the vitamin B_{12} excretions had decreased for all subjects and there was a tendency toward uniform output. In the last metabolic period, after the diet had been supplied for 42 days, the excretions ranged from 0.02 to 0.03 μg per day with less than a twofold variation. In the first metabolic period following the self-chosen diet, the average amount of vitamin B_{12} excreted was 9% of the dietary intake, and in successive periods 3 to 4%.

There was no measurable vitamin B_{12} in the urine for each of 4 girls in period 5 who had received the antibiotic, Cosa-Tetracyn,[2] during this period or in the preceding period. Similar findings had been noted for folic acid when microbiological determinations were made using the test organism *Streptococcus faecalis*.[3] Further investigations indicated that the apparent absence of folic acid was attributable to inhibition of the test organism *S. faecalis* by Cosa-Tetracyn excreted in the urine rather than to lowered excretion of folic acid. A similar explanation may apply to the vitamin B_{12} analyses reported here.

Chow ('51), in a study with normal children, reported no detectable vitamin B_{12} activity in the urine even after daily oral supplements of 25 μg of vitamin B_{12}. Studies with normal adults consuming ordinary diets have shown urinary excretions of vitamin B_{12} which ranged from 0.02 to 0.27 μg per day (Register and Sarett, '51; Mollin and Ross, '52).

The low excretion of vitamin B_{12} in the 1958 study, when the intakes were between 0.68 and 1.15 μg daily, may indicate that practically the full amount of these intakes was needed for cellular maintenance and tissue growth leaving almost none to be excreted unused. This suggests the possibility that an intake of 1 μg of vitamin B_{12} daily may closely approach the minimal requirement for these children.

Diets 2, 3, 4, and 5 were patterned to include largely the usual kinds and amounts of foods needed to meet the Food and Nutrition Board's ('53) recommended allowances for children 7 to 9 years of age, except that protein was above the recommended allowance in diets 3 and 5. Since these diets supplied between 3.9 and 4.5 μg of vitamin B_{12} per day, it could be assumed that the usual nutritionally adequate diet for this age of child would supply about 4 μg of vitamin B_{12} daily.

SUMMARY

The vitamin B_{12} content was determined for each of 6 controlled diets used in a series of metabolic studies with a total of 23 healthy 7- to 9-year-old girls. Urinary vitamin B_{12} excretions were determined for 11 girls receiving two of these diets that were low in protein.

Diets with a daily average of 48 to 88 gm of total protein (about 65% from animal sources) contained from 3.9 to 4.5 μg of vitamin B_{12} per day. Diets of lower protein content with a daily average of 18 and 22 gm of total protein (about 40% from animal sources) contained 0.7 and 1.1 μg of vitamin B_{12} per day, respectively.

The urinary excretion of vitamin B_{12} for the girls receiving low-protein diets was 9% of the dietary intake at the initiation of the controlled diet phase of the study and from 3 to 4% in latter phases.

Diets, made up of ordinary foods patterned to meet the Food and Nutrition Board's recommended allowances for children 7 to 9 years of age, supplied about 4 μg of vitamin B_{12} daily.

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Effect of Vitamin B_{12} on Wound Healing.* (20156)

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Vit. B_{12} has been found to be an important growth factor in young animals(7,8,11) and several investigators believe the vitamin participates in protein synthesis(2,5,13,14). Numerous studies have emphasized the significance of body proteins in wound healing (1,3,9,10,12). It seemed appropriate, therefore, to investigate the role of B_{12} in wound healing of animals with normal and depleted protein stores.

Method. Sherman rats weighing about 300 g were used. They were fed Rockland Chow diet prior to the study and under observation they were given one of 3 diets for 3 weeks before wound infliction and for 6 days post-operatively. A synthetic diet containing 19% protein originally used by Darrow(4) was given to one group of the animals. This was composed of: lactalbumin 18, Crisco 22, dextrin 32, sucrose 25, cod liver oil 1, powdered yeast 2, bone ash 2, NaCl 1, and KH_2PO_4 2 g. The second group received a 0.9% low protein diet containing the same components except that lactalbumin was omitted and the proportions of Crisco, dextrin and sucrose were increased to 26, 36 and 29 g respectively. Both these synthetic diets had approximately the same caloric value of about 500 calories

TABLE I. Weight Changes of Animals on Specified Diets and Medication* before and after Operation

Diet†	Medication	No. of rats in group	Wt before diet	Wt after diet for 3 wk	Avg daily intake (g)	% wt change on diet	Wt at death	Avg post-operative daily intake	% wt change since operation
Balanced synthetic	Saline for 6 p‡ days	6	313	358	15.6	+14.4	343	15.0	—4.2
"	Saline 21 pr and 16 p days	6	306	344.3	17.7	+12.5	345	16.7	+ .7
Rockland chow	Saline 21 pr and 6 p days	10	307	331	18.6	+ 7.8	321	16.1	—3.0
Balanced synthetic	Vit. B_{12} 6 p days	10	301	339	15.7	+12.3	331	8.9	—2.4
"	Vit. B_{12}, 21 pr and 6 p days	8	279	312	13.5	+11.9	305	9.3	—1.9
Rockland chow	Vit. B_{12} 6 p days	10	322				325		+ .9
Low protein	Saline 6 p days	17	324	279	12.6	—13.8	278	11.5	— .4
"	Saline 21 pr and 6 p days	11	328	275	14.2	—16.4	270	11.6	—1.9
"	Vit. B_{12} 6 p days	14	296	252	10.9	—14.9	241	7.2	—4.0
"	Vit. B_{12} 21 pr and 6 p days	8	303	258	11.3	—14.8	250	8.9	—3.1

* Rats given vit. B_{12} received .5 μ/100 g body wt/day. Daily dosage of isotonic saline to all animals was $\frac{\text{wt of rat}}{200}$.

† All specified diets given 3 wk before operation and 6 days post-operatively.

‡ p = post-operation; pr = pre-operation.

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TABLE II. Data to Determine Effect of Vitamin B_{12} on Tensile Strengths of Wounds in Rats Fed Control and Low Protein Diets.

	Balanced synthetic diet			Rockland chow diet	Low protein diet			Balanced synthetic minus low protein diets		Rockland chow–low protein diets
	Vit. B_{12} or saline, 21 pr* & 6 p days (1)	Vit. B_{12} or saline, 6 p days (2)	Diff. between pr & p, & only p admin. (1)–(2)	Vit. B_{12} or saline, 6 p days (3)	Vit. B_{12} or saline, 21 pr* & 6 p days (4)	Vit. B_{12} or saline, 6 p days (5)	Diff. between pr & p, & only p admin. (4)–(5)	Vit. B_{12} or saline, 21 pr* & 6 p days (1)–(4)	Vit. B_{12} or saline, 6 p days (2)–(5)	Vit. B_{12} or saline, 6 p days
Vit. B_{12}										
Avg tensile strength	125.2	133.3	—8.1 (b)	129.5	82.0	89.4	—7.4 (b)	43.2 (a)	43.9 (a)	40.1 (a)
No. of rats	7	7		10	8	10				
Infection rate (%)	0	0		29	22	50				
Saline controls										
Avg tensile strength	102.0	111.9	—9.9 (b)	108.2	76.5	78.4	—1.9 (b)	25.5 (a)	33.5 (a)	29.8 (a)
No. of rats	6	7		10	7	15				
Infection rate (%)	0	16.6		20	54.5	39				
Difference (Vit. B_{12} — saline)	23.2 (a)	21.4 (a)		21.3 (a)	5.5 (b)	11.0 (b)				

Note: All wounds tested on 6th p day. Designated diets given for 21 pr and 6 p days. Vit. B_{12} dosage 0.5 μ/100 g body wt/day. Isotonic saline dosage was wt of rat/200. Student's t test at .01 levels used to determine significance. (a) designates that the difference is statistically significant. (b) the difference is not statistically significant.

* pr = pre-operative; p = post-operative.

per 100 g of diet. The rest were maintained on the Rockland Chow diet said to contain 25% protein by analysis and with a caloric value of 320 calories per 100 g of diet. The weights of the animals were recorded daily and average daily intakes of food were determined. Percentages of weight change during the pre and postoperative periods were calculated (Table I). The rats on all these diets were given subcutaneously 0.5 μ of vit. B_{12} per 100 g of body weight in saline daily, while the controls received a comparable amount of saline daily. These solutions were given to some animals for the entire experimental period and to others only following operation (Table II). Except as noted later, all rats were killed on the sixth postoperative day. At this time the incidence of wound infection was noted (Table II). The wounds of the non-infected animals were excised, divided into 1 cm strips and tested for tensile strength as previously described(6). From each wound at least 3 segments were tested by a tensionmeter and the results were measured in grams. The strengths of all strips taken from a wound were totaled and divided by the number of segments. This gave the average tensile strength of the wound per cm. Sections were taken for microscopic study.

Results. All rats gained weight on the balanced synthetic and Rockland Chow diets and those fed low protein diets for 3 weeks invariably lost weight. Giving vit. B_{12} neither increased the food intake nor did it alter the weight changes in any group. This was evident in both the pre and postoperative periods (Table I).

Vit. B_{12} given either before and after operation or only postoperatively caused an increase in the tensile strength of sixth day wounds in rats fed the synthetic diet containing 19% protein, and the same effect was noted when B_{12} was given postoperatively to Rockland Chow fed animals. No difference was observed in the average tensile strengths for groups given the vitamin both before and after surgery, or only after operation. The tensile strengths of wounds in animals maintained on the low protein diet showed a significant retardation of wound healing when compared to those fed the 19% or 25% protein diets. The administration of B_{12} to the low

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protein rats caused no significant increase in the strengths of their wounds. For results of statistical analysis of the above differences, see Table II. The low protein rats in general had a high incidence of wound infection by gross examination. (Table II). Vit. B_{12} did not check this complication.

Sections of the wounds studied by microscopy showed the usual findings for normal and low protein fed animals. The latter showed diminished fibroplasia, derangement of the normal polarity of the fibroblasts and evidence of tissue edema. The administration of vit. B_{12} did not cause microscopic changes in the rats of any group.

Since vit. B_{12} when given to rats maintained on balanced diets increased on the average the tensile strength of six-day-old wounds, it seemed important to know when this increased strength was acquired. Accordingly, studies were made on wounds three to eight days old in rats fed balanced synthetic or Rockland Chow diets. Postoperatively they were given parenteral vit. B_{12} or saline from the time of operation until death. With the animals on these diets, statistical analysis did not show any difference in the average change of tensile strength from the third to the eighth day when comparing the vitamin groups with the saline controls (Table III). It was determined, however, that the average tensile strength was significantly higher on the third day in the vitamin fed groups. Thus the stronger wounds previously noted at the sixth day in rats fed B_{12} could possibly be explained by greater acquisition of tensile strength by the third day. This would indicate that the latent period of healing had been shortened in the rats given B_{12}. The continued greater acquisition of strength, on the other hand, was not evident by the eighth day when the vitamin and saline groups were compared. In these two groups, more extensive data will be necessary to detect a significant difference in the change of average tensile strength from the third to the eighth day.

TABLE III. Effect of Vit. B_{12} on Mean Tensile Strength of Wounds in Rats Fed Balanced Diets from Third to Eighth Day (See Text).

	Rockland chow diet		Balanced synthetic diet	
Post-op days tested	No. tested	Tensile strength (g)	No. tested	Tensile strength (g)
		Vitamin B_{12}*		
3	3	54.9	4	51.8
4	4	68.7	4	60.7
5	6	80.8	5	96.6
6	10	129.5	7	133.3
7	7	152.4	6	139.8
8	5	170.0	5	173.5
		Saline†		
3	3	39.7	4	42.6
4	3	62.0	6	60.3
5	5	80.7	5	81.8
6	10	108.2	7	111.9
7	5	140.0	6	127.4
8	5	159.7	5	179.6

* 0.5 μ/100 g body wt/day.

† Dosage of isotonic saline was $\frac{\text{wt of rat}}{200}$.

Respective diets were given 3 wk before surgery and postoperatively until animal was killed.

Summary. 1) These data show that B_{12} increased the tensile strength of wounds during the early phases of healing in rats fed balanced diets containing 19% or 25% protein. This effect was evident at least by the third day in the wounds studied, and it was most noticeable by the sixth day. 2) The wounds that were studied in the saline control and vitamin treated rats from the third to the eighth day showed no significant difference in their healing rates at the eighth day. 3) The delayed healing rate and increased incidence of wound infection in rats that were protein depleted by diet were not significantly altered by administration of vit. B_{12}.

Grateful acknowledgment is made to Phyllis B. Michelsen, Division of Biostatistics of the Faculty of Medicine, Columbia University, for the statistical analyses. The vitamin B_{12} used in this experiment was donated by Merck, Inc., Rahway, N. J.

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Parathyroid Extract and Alkaline Phosphatase Activity.* (20157)

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Parathyroid extract has been reported to increase(1) and to have no effect(2) on the alkaline phosphatase activity of the kidneys of rats. This difference now has been resolved by repeating the experiments with each of the separate laboratories performing simultaneously both of the previously reported procedures.

Results. The results obtained in both laboratories were identical (Table I). The parathyroid extract‡ produced an increase in phosphorus excretion, but no change in the

TABLE I. Inability of Parathyroid Extract to Change Alkaline Phosphatase Activity of Kidney.* (5 male rats per group.)

Treatment	B. wt, g	Urine P, mg (24 hr)	Kidney, g	Alkaline phosphatase, U/g — Homogenate at 5°, 18 hr† — K–A pH 9.7	K–A 8.6	Bodansky 9.7	Bodansky 8.6
			Bonelli and Sala				
Control	215 ±10‡	5.1 ± 1.7	1.64 ± .10	56 ± 3.4			35 ± 2.2
PTH 1 ml	200 ± 6	9.6 ± 1.5	1.52 ± .05	54 ± 6.4			32 ± 1.7
			Kochakian and Reed				
Control	209 ± 6	7.7 ± 1.7	1.52 ± .07	111 ± 5.0	46 ± 4.9	36 ± 5.2	19 ± 2.1
PTH 2 ml	210 ± 6	24.1 ± 2.6	1.51 ± .04	113 ± 4.4	46 ± 2.9	34 ± 3.5	18 ± 1.3
				Homogenate at 23–30°, 42 hr† — K–A pH 9.7	K–A 8.6	Bodansky 9.7	Bodansky 8.6
			Bonelli and Sala				
Control	215 ±10‡	5.1 ± 1.7	1.64 ± .10	18 ± 4.2			18 ± 4.2
PTH 1 ml	200 ± 6	9.6 ± 1.5	1.52 ± .05	19 ± 2.2			13 ± 3.4
			Kochakian and Reed				
Control	209 ± 6	7.7 ± 1.7	1.52 ± .07	89 ± 3.9	36 ± 2.1	29 ± 3.1	25 ± 1.7
PTH 2 ml	210 ± 6	24.1 ± 2.6	1.51 ± .04	75 ± 4.0	34 ± 5.7	27 ± 4.9	22 ± 2.4

* Autopsy 6.5 hr after inj. of parathyroid extract.
† Temperature and duration of autolysis.
‡ Stand. error of the mean, $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

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‡ The parathyroid extract was provided by Eli Lilly and Co.

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Nature of the Relationship between Vitamin B_{12} Binding and Cell Uptake[1]

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Transcobalamin II was shown to be a specific B_{12} transport protein promoting the uptake of B_{12} by human normal and malignant cells. Transcobalamin I, the other known transport protein, and the B_{12} binding substances of erythrocytes, leukocytes, saliva, and gastric juice, did not enhance the uptake of B_{12} by tissue cells *in vitro*. Transcobalamin II was strongly retained on carboxymethyl-cellulose, but the other B_{12} binders passed through with the equilibrium buffer. After uptake by human tumor cells a transcobalamin II-B_{12} complex was changed into a macromolecule that had the chromatographic characteristics of transcobalamin I.

Two trace proteins are known to participate in the transport of vitamin B_{12} in normal plasma. Their properties are listed in Table I.

Protein bound B_{12} has been shown to be of greater physiological significance than B_{12} alone. Callender and Lajtha (11) reported that B_{12} bound to normal gastric juice or normal serum could ripen megaloblasts from pernicious anemia (PA) bone marrow *in vitro*. This was confirmed by Astaldi and Cardinali (12) and by Pendl and Franz (13), whereas Swan *et al.* (14) failed to reproduce these results. Cooper and Paranchych (15) demonstrated that human serum, mouse serum, and ascitic fluid enhanced the uptake of B_{12} by mouse Ehrlich ascites cells. Only human serum promoted the uptake of cyanocobalamin by HeLa cells. Subsequently, it was demonstrated that only those cobamides containing the benzimidazole moiety in the nucleotide portion of the molecule and unsubstituted amides in the corrin nucleus were taken up by Ehrlich mouse ascites cells (16). Sarcoma 180 cells will take up B_{12} bound to mouse serum and ascites fluid but not mouse stomach extract (17). The uptake of B_{12} by erythrocytes is enhanced by human plasma and increases as the percentage of reticulocytes increases (18). In a classic experiment (19, 20) Castle proved the need for intrinsic factor binding of B_{12} for intestinal absorption. The preferential absorption of intrinsic factor bound B_{12} by intestinal tissue *in vitro* has been demonstrated in a number of systems (21–24).

Rat liver slices, perfused rat liver, and human liver slices have been shown to take up B_{12} in the presence of intrinsic factor much more readily than B_{12} alone (25–27).

Our previous *in vivo* studies (6, 8, 28) and the *in vitro* work of others cited above suggested specific functions of transcobalamin I (TC I) and transcobalamin II (TC II) in respect to uptake of vitamin B_{12} by the cell. Therefore, the present study was designed in order to evaluate each protein by a simple *in vitro* system of HeLa cells grown in monolayer culture. Since there is some evidence of similarity of B_{12} binders in serum, leukocytes, erythrocytes, saliva, and gastric juice (29), they too were tested in this system. Preliminary reports have been presented (30, 31).

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TABLE I

PROPERTIES OF TRANSCOBALAMIN I AND II

Property	TC I	TC II
Mobility on paper electrophoresis pH 8.6, veronal buffer.	α (1-3)	β (4)
Mobility on starch and geon block electrophoresis pH 4.5 acetate buffer.	Anodal (5, 6)	Cathodal (5, 6)
DEAE column	Post-albumin (7, 8)	Pre-albumin (8)
CM column	Eluted early (8)	Retained (8)
Plasma removal	Slow (6, 8)	Fast (6, 8)
Nature	Seromucoid (5, 6, 9)	Seromucoid (6)
Transport function	Carries the endogenous B_{12} (8)	Transport of B_{12} when first injected or ingested (8, 10)

MATERIALS AND METHODS

Heparinized plasma was obtained from healthy laboratory personnel. TC I, which carries the endogenous B_{12}, is almost completely saturated in normal plasma. In chronic myelogenous leukemia (CML), and to a lesser extent in untreated pernicious anemia (PA), additional B_{12} can be bound to the serum (32). TC II is decreased or blocked in CML in relapse, and treatment with Busulfan was shown to increase the amount of B_{12} bound to it (28). For these reasons CML and PA plasma were used in some studies. Plasma from patients with CML in relapse and remission was obtained from the National Cancer Institute through the generosity of Dr. Paul Carbone, and some from our own institution. Erythrocytes, leukocytes, and saliva were obtained from healthy donors. Erythrocytes were washed three times with saline after removal of the buffy coat. A suspension containing 5×10^9 cells/ml was prepared and the cells were broken by alternate freezing and thawing in an ultrasonic generator (33). Leukocytes were obtained by the method of Dioguardi (33), which gave a preparation of 65-80% granulocytes. A suspension containing 10^7 cells/ml was prepared and was usually broken up in the manner of erythrocytes, although one preparation was broken by pressure of 10,000 psi in a French Pressure Cell. Saliva was produced by chewing blocks of paraffin wax and was collected in an ice-cooled beaker. The preparation was centrifuged at 4° at 3000g for 20 minutes prior to use and stored in the frozen state. Gastric juice, macroscopically free from bile and blood, was obtained from normal patients. Pepsin was inactivated by raising the pH to 10 for 30 minutes with 10% KOH. The pH was then reduced to 7.0 with 0.1 N H_2SO_4 and the preparation was stored in the frozen state. ^{57}Co-labeled B_{12} of specific activity 70-150 μC/μg was obtained from Radiocentre, Amersham, England, through the courtesy of Merck Chemical Division, Merck and Co., Inc. ^{57}Co-labeled B_{12} of activity of 16 μC/μg, obtained from Merck & Co., Inc., Rahway, New Jersey, was used in some studies. USP reference standard nonradioactive B_{12} was used to reduce the specific activity when large amounts of B_{12} were added.

TABLE II

AMOUNT OF B_{12} ADDED AND PERCENTAGE BOUND

Radioactive B_{12} was added to the various preparations as indicated, and the percentage of binding was measured after dialysis at 4° for 24 hours against three changes of phosphate buffer, pH 8.0, 0.01 M.

Preparation	Concentration, cells/ml	Amount added (ng/ml)	% Bound
Erythrocytes	5×10^9	2	81
Leukocytes	1×10^7	10	91
Saliva		10	95
Gastric juice		10	96
Plasma		0.3	90-100

The amount of B_{12} added and the percentage bound are listed in Table II.

Visking dialysis tubing obtained from Union Carbide Corp., Chicago, was used for dialysis and concentration of fractions by ultrafiltration. It was cleaned with 0.15% disodium versenate as suggested by Grasbeck (34). DEAE-cellulose, type 40, 0.92 meq/gm, was obtained from Schleicher and Schuell. All the B_{12} binders were fractionated in a like manner using a convex gradient of falling pH and rising molarity. The radioactive B_{12} peak from this step was then fractionated on CM-cellulose, 0.7 meq/gm (Whatman), with equilibration buffer (acetate) at pH 5.4 (0.02 M) followed by a gradient, and finally

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phosphate buffer at pH 7.8 (0.2 M). These procedures have been described in detail (8). Protein was estimated by the method of Lowry *et al.* (35). 199 medium and calf serum was obtained from Grand Island Biological Co., Grand Island, New York. Primary human amnion cells were purchased from Flow Laboratories, Rockville, Maryland. Primary human kidney cells were obtained from Microbiological Associates, Bethesda, Maryland. HeLa cells were grown for the first 2 days after seeding in a medium consisting of 199 with 20% calf serum, and for the next 2 days a medium containing 10% calf serum was substituted. Except when used in short-term uptake studies, 100 units/ml of penicillin-streptomycin and 25 units/ml Mycostatin were added to all media. Cells were grown in monolayer culture and uptake studies were performed *in situ*. Monolayer cultures were used in the uptake studies because the fixation of proteins to damaged cells has been reported (36-38). Manipulation was kept at a minimum to obviate this artifact. Prior to B_{12} uptake the cells were rinsed with buffered saline containing 0.1% dextrose. Material from the CM-cellulose peak of the B_{12} binding substance to be studied was dialyzed against buffered saline and then added to the monolayer culture together with the 199 medium. Cells were incubated at 37° for 2-3 hours without shaking. At the end of this period the medium was poured off, the monolayer was rinsed with cold, buffered saline, and the cells were removed with a rubber-tipped spatula. They were then washed three times with ice-cold buffered saline. Cells were counted in a hemocytometer and radioactivity was measured in an automated, well-counting system that was connected to a single channel scintillation spectrometer. Counting efficiency was 1.5×10^6 cpm/μC, and background was between 30 and 40 cpm. A counting error of 5% or less was obtained.

In order to determine the fate of the TC II-B_{12} complex after uptake, HeLa cells were incubated in 199 medium containing 20% normal human serum that had 300 pg $^{57}CoB_{12}$/ml bound to it. At this concentration binding is almost exclusively by TC II (32). After 48 hours the medium was poured off and the cells were washed three times with cold 199 medium using a sterile technique. A medium consisting of 199 and 20% normal human serum without any added B_{12} was substituted for another 48 hours. At the end of this period the medium was poured off, concentrated by ultrafiltration, dialyzed against starting buffer, and chromatographed on DEAE-cellulose. This was followed by chromatography of the ^{57}Co fraction on CM-cellulose.

RESULTS

The location of the DEAE-cellulose chromatographic peaks of TC II (between beta globulin and albumin) and TC I (post-albumin) were as reported previously (8). TC I from the DEAE column was eluted early from the CM-cellulose column, but TC II was retained until 0.2 M phosphate buffer, pH 7.8, was used (8). In all of the studies reported below the TC I and TC II used came from CM-cellulose chromatography of the DEAE column fractions. This gives a TC II of relatively high purity (8). TC I can be further purified by hydroxylapatite column chromatography (31), but this was not considered to be necessary for the present study.

Figure 1 shows the uptake of TC I- and TC II-bound B_{12} by a monolayer of HeLa cells. The rapid uptake of TC II was similar to the *in vivo* uptake by liver of TC II injected into a normal person (Fig. 2). Since the TC I was derived from leukemia plasma, there remained the possibility that a lack of some normal constituent of plasma could explain the poor uptake of TC I-B_{12}. Another possibility was that leukemia plasma contained an inhibitory substance which was not removed during the fractionation. Labeled TC I and TC II were separated from the plasma of a patient with treated CML who was in remission and therefore had both proteins (Fig. 3a). The HeLa cell uptakes are shown in Fig. 4, and demonstrated that (*a*) the leukemia TC II was

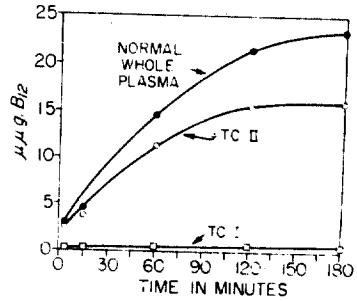

FIG. 1. Uptake of TC I-$^{57}CoB_{12}$ and TC II-$^{57}CoB_{12}$ by HeLa cells in monolayer as $\mu\mu g$ (pg) of $B_{12}/10^6$ cells. The TC I curve is a composite of three experiments and that of TC II of six experiments.

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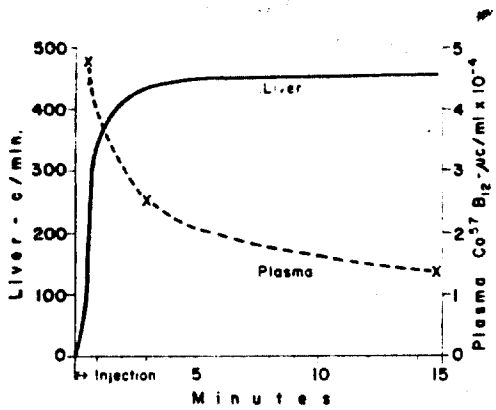

FIG. 2. Uptake (by continuous external counting) of intravenous TC II-$^{57}CoB_{12}$ by intact liver. [Lot 6 from previous study (8)]. The simultaneous disappearance of the TC II-$^{57}CoB_{12}$ from plasma is superimposed. Liver uptake was measured by a collimated probe attached to a rate meter. The count rate was continually recorded on a Texas Instrument Recti-Riter. The figure shows a smooth curve drawn through the actual trace. The y-axis gives the counts/minute for the area under collimation.

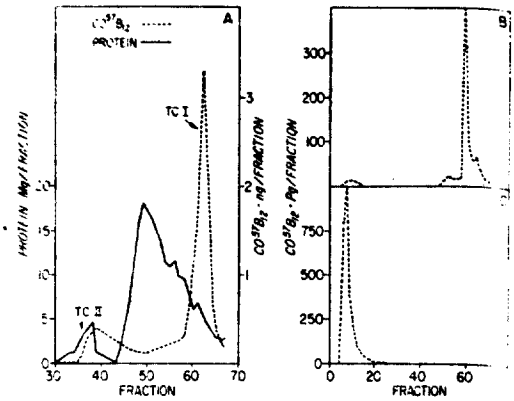

FIG. 3. (A) DEAE-cellulose fractionation of 57 ml of chronic myelogenous leukemia plasma after 300 pg $^{57}CoB_{12}$ had been added per milliliter. (B and C) CM-cellulose chromatography of TC II (B) and TC I (C) peaks from (A).

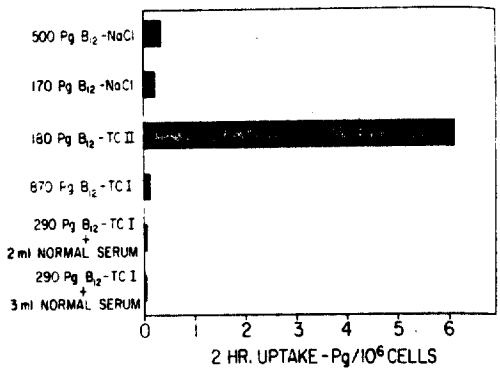

FIG. 4. HeLa cell uptake of CM fractions from plasma of Fig. 3.

taken up, (*b*) the leukemia TC I was not, and (*c*) the addition of normal serum to the TC I-B_{12} complex did not enhance uptake. Figure 4 also shows the failure of the cells to take up $^{57}CoB_{12}$ in saline. In an experiment not illustrated, CML plasma was added to normal TC II-B_{12} and failed to inhibit uptake. Since PA plasma, like CML plasma, but in contrast to normal plasma, contains some unsaturated TC I, the experiment was repeated with plasma from this source. The labeled TC I and TC II were separated by the methods of Fig. 3. $^{57}CoB_{12}$, 83.8 pg, and bound to TC I, when incubated with HeLa cells for 3 hours gave an uptake of 0.03 pg per 1×10^6 cells; when 77.5 pg $^{57}CoB_{12}$ bound to TC II was similarly treated 4.56 pg were taken up per 1×10^6 cells. The HeLa cells took up the TC II but not the TC I.

These studies with TC I and TC II showed that (*a*) binding of the B_{12} to TC II promoted uptake by the cells, and (*b*) the difference in uptake of TC I and II was a function of their nature and not of the source of the plasma.

In two preliminary studies using primary cultures of human amnion cells and human kidney cells, TC II was taken up while TC I was not.

The $^{57}CoB_{12}$-labeled binders from leukocytes, saliva, erythrocytes, and gastric juice were separated by the technique used for TC I and TC II. The results of the DEAE-cellulose column chromatography were identical to those of Simons (29). The results of the CM-cellulose chromatography are shown in Fig. 5. Like TC I, but in contrast to TC II (Fig. 3), none of these binders was retained. The CM-cellulose fraction of the gastric juice binder presumably contains both "R" (gastric binder with rapid electrophoretic mobility) and "S" (gastric binder with slow mobility), as they have been shown

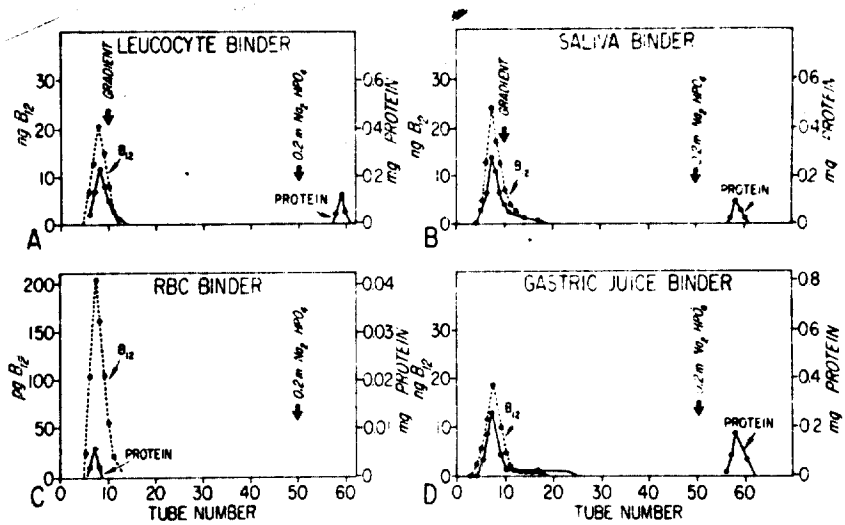

FIG. 5. Elution of four cell and body fluid B_{12} binders from CM-cellulose columns. In each case the $^{57}CoB_{12}$ peak from a DEAE-cellulose column was applied to the CM-cellulose column.

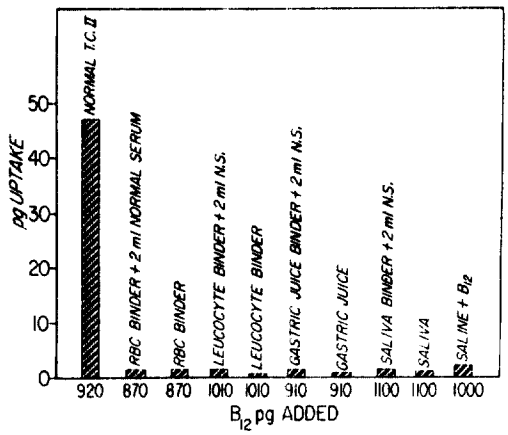

FIG. 6. HeLa cell uptake of protein-$^{57}CoB_{12}$, pg/10^6 cells, of the binders illustrated in Fig. 5 and compared with normal TC II-$^{57}CoB_{12}$.

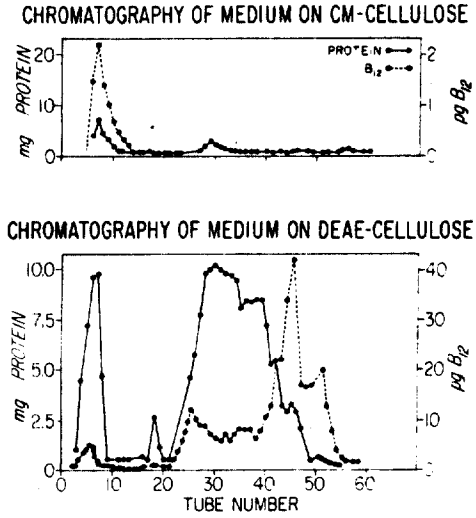

FIG. 7. Isolation of $^{57}CoB_{12}$-protein complex from the medium which had contained HeLa cells that previously took up TC II-$^{57}CoB_{12}$ from a completely separate medium.

to come through with the equilibration buffer on CM-cellulose chromatography (34). The HeLa cell uptakes are shown in Fig. 6; only TC II was taken up. The addition of normal serum to any of the complexes did not promote uptake.

In a final experiment HeLa cells were exposed to TC II-$^{57}CoB_{12}$ for 48 hours, and the medium was replaced by one containing normal human serum but no added B_{12}. After 48 hours the medium was collected and processed in the usual way. The protein $^{57}CoB_{12}$ complex behaved like TC I-B_{12} (Fig. 7). This suggestion that TC I is an exit carrier in contrast to the function of TC II in B_{12} uptake by the cell will be discussed below.

DISCUSSION

The results of protein-bound B_{12} uptake by human tumor cells and of CM-cellulose chromatography indicate that TC II is a specific B_{12} transport substance which does not appear to be identical with the B_{12} binders in erythrocytes, leukocytes, saliva, and gastric juice. This does not preclude the possibility of shared characteristics such as a common antigenic component or similar electrophoretic mobility.

The *in vivo* uptake of B_{12} bound to plasma protein is thus indicative of TC II binding since TC I has been shown to be nonfunctioning in the system described. The possibility exists that this approach may be used to measure the amount of TC II binding in CML or PA or in other diseases where an abnormal binding pattern is known to exist. The two plasma binders which are known to participate in the *in vivo* B_{12} transport were isolated and partially purified. Complete isolation is hampered by the fact that these proteins occur only in trace amounts, and as purification proceeds the B_{12} molecule tends to dissociate from the complex. Other investigators have encountered the same problem (39).

The similar behavior of the two primary cultures of normal human cells with regard to the selective uptake of TC II-bound B_{12} further supports the view that the findings may be of physiological significance.

TC II was the only plasma B_{12} binder retained on CM cellulose, which is characteristic of proteins with a net positive charge. Similarly, the mouse ascitic fluid and mouse serum B_{12} binders which are retained on CM-cellulose promoted the uptake of B_{12} by sarcoma 180 cells (17). Evidence has been presented that a B_{12} binding protein which is retained on CM-cellulose is present in a number of animal sera (40). Recently (41) it was reported that basic polyamino acids and proteins are taken up by cells in tissue culture at a rate as much as 3000 times greater than serum albumin. Arginine-rich histones were considerably more active than lysine-rich histones. Uptake of a B_{12}-binding protein is believed to consist of a primary stage in which physicochemical adsorption of cyanocobalamin to the surface of the cell takes place; this is followed by a secondary phase in which the transport of the B_{12} across the cell membrane occurs (42). Tumor cells carry a high surface negative charge, as measured by cell electrophoresis, due to the presence of sialic acid on the cell surface (43-45). It may be speculated that the specificity of uptake of TC II-bound B_{12} is governed not only by the presence of distinct receptor sites on the cell surface but also by the actual configuration and net charge of the carrier protein delivering the B_{12} molecule to the site of absorption.

The phenomenon of B_{12} being transported into a cell by one protein and exiting via another is paralleled by the work of Bosse and Wilson (46), who demonstrated that an intrinsic factor-B_{12} complex taken up by intestinal epithelial cells is modified into another protein-B_{12} macromolecule, which is then excreted into the lymphatics and circulation. Miller and Sullivan (47) demonstrated the presence of sialic acid in their B_{12} binding protein (B_{12}BP) which would appear to be identical with our TC I. This lends support to the hypothesis (48) that most excreted proteins are glycoproteins, wherein the carbohydrate unit serves as a passport for the exit of the protein from the cell. The conversion of TC II-B_{12} complex into a complex behaving like TC I may be due to a number of causes: (*a*) Possibly the TC II-B_{12} complex undergoes some structural alteration during one of the phases of uptake and assumes the configuration and characteristics of TC I. (*b*) Alternately the B_{12} molecule may be released from the complex and *de novo* synthesis of a TC I-like substance may occur, probably at the cell membrane which binds the B_{12} molecule. (*c*) Finally, one may postulate that the TC II-B_{12} complex enters the HeLa cell intact and remains so to combine with a protein present to form a new macromolecule; this is suggested by the fact that TC I has a molecular weight roughly three times that of TC II (49). Investigations to explore these alternatives are underway.

Addendum. Since submission of this report a paper by Kai Simons, Gosta Kvist, and Maria Sauren-Lindfors has been published in *Ann. Med. Exptl. Fenn.* **44**, 361 (1966). These authors studied the uptake of TC II (their serum R) and other binders by two systems of rat liver and guinea pig intestinal homogenates.

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STUDIES ON THE ABNORMAL HIGH BINDING CAPACITY OF BLOOD FOR VITAMIN B_{12} IN CHRONIC MYELOID LEUKEMIA

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(Received March 29, 1971)

SUMMARY

The serum vitamin B_{12} values have been assayed in 47 patients with chronic myeloid leukemia, and were estimated to be above 2000 pg/ml in 91% of the patients. The vitamin B_{12} binding capacity was further investigated on leucocytes isolated from normal individuals and from patients with chronic myeloid leukemia. By means of agar-gel micro-electrophoresis and immunoelectrophoresis of a leucocyte extract antigenic proteins were traced. Although the electrophoretic pattern of leukemic leucocytes showed more fractions than normal leucocytes, the immunoelectrophoretic pattern of all samples of leucocyte extracts incubated with radio-active vitamin B_{12} revealed only one vitamin B_{12} binding protein. Thus, in chronic myeloid leukemia as well as in normal leucocytes, only one vitamin B_{12} binding protein with α-globulin-mobility occurred, supporting the idea that in chronic myeloid leukemia an increased number of leukemic leucocytes will liberate the vitamin B_{12} binding protein to the blood stream rather than liberating an abnormal leukemic protein with high vitamin B_{12} binding capacity. No significant correlation was found between the number of leucocytes and the levels of vitamin B_{12} in serum.

INTRODUCTION

Beard, *et al.*[1] demonstrated that the level of serum vitamin B_{12} was increased in patients with chronic myeloid leukemia. This has later been confirmed by several groups[2,3] and we have demonstrated that the mean value of serum vitamin B_{12} level was above 2000 pg/ml serum in 91% of patients with chronic myeloid leukemia[4]. In normal serum there seems to be 2 vitamin B_{12} binding proteins, an α_1-globulin which is regarded as a circulating depot of vitamin B_{12}, and a β-globulin which seems to transport the serum vitamin B_{12}. The α-globulin may correspond to Hall's transcobalamin I and the beta-globulin to Hall's transcobalamin II[5].

In chronic myeloid leukemia a greater amount of vitamin B_{12} is found to be bound to the α_1-globulin fraction than in normal serum, and it has been suggested

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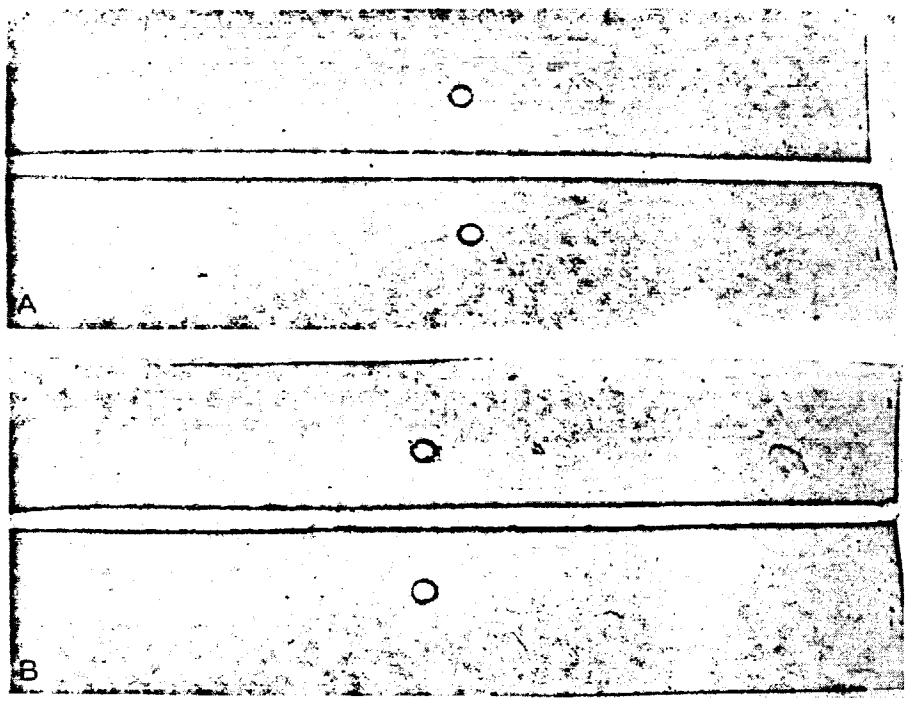

Fig. 5. Immunoelectrophoresis of leucocyte extract from normal leucocytes (A) and from leucocytes from patients with chronic myeloid leukemia (B).

DISCUSSION

The present communication confirms that in patients with chronic myeloid leukemia, the serum B_{12} values are increased in most patients.

The present findings of one vitamin B_{12} binding protein in leucocyte extract are in agreement with the data of Simon and Weber[6] and Gottlieb *et al.*[7], and support the idea that the high serum vitamin B_{12} value in case of chronic myeloid leukemia is due to the high number of myeloid cells in the peripheral blood. This is further supported by the demonstration in the present publication that during blast crisis, the serum vitamin B_{12} is decreased. Thus, it must be imagined that the leukemic leucocytes are able to liberate the vitamin B_{12} binding protein to the blood stream.

The demonstration of Kidd and Thomas[19] that there were no data supporting the idea of a specific vitamin B_{12} binding protein in chronic myeloid leukemia is quite in agreement with our data demonstrating by a combination of immuno-electrophoresis and autoradiography that there was only one α-globulin binding vitamin B_{12} in chronic myeloid leukemic leucocytes and this precipitating protein was identical to that found in normal leucocytes.

Thomas and Anderson[20], and Meyer[21], Meyer *et al.*[22] have elucidated the binding capacities of leucocytes for vitamin B_{12}. The latter demonstrated a two-phase binding, probably due to the presence of two vitamin B_{12} binding proteins. This has, however, not been confirmed by our data which only showed one serum vitamin B_{12} binding

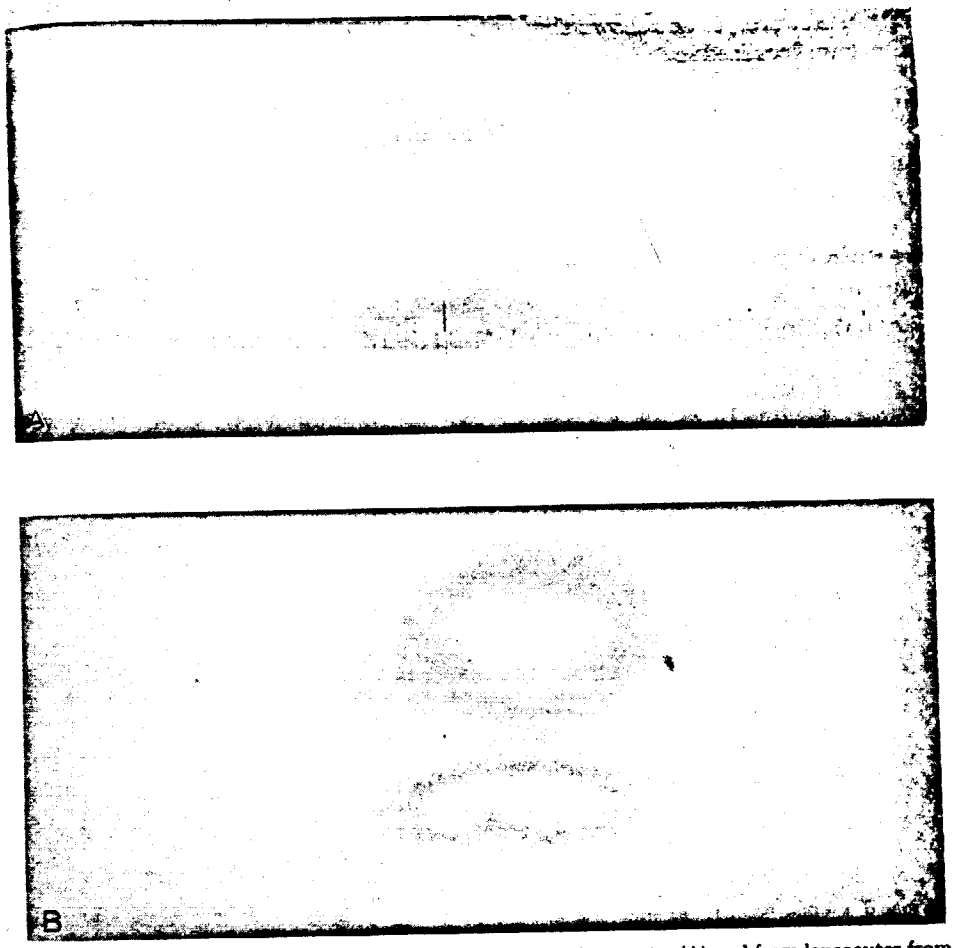

Fig. 6. Autoradiography of leucocyte extract from normal leucocytes (A) and from leucocytes from patients with chronic myeloid leukemia (B). The leucocyte extract was mixed with Co^{58} labelled vitamin B_{12}. After immunoelectrophoretic separation the slides were placed on ordinary X-ray film.

TABLE III

PERCENTAGE DISTRIBUTION OF ^{58}Co RADIOACTIVITY OF VITAMIN B_{12} IN LEUCOCYTES

	Sample No.	α-globulin %	β-globulin %	γ-globulin %
Normal	178	90		10
	177	61	39	
	179	85	15	
Chronic myeloid leukemia	216	24	17	59
	213	100		
	214	49	11	40

The immuno-electrophoretic pattern was cut out in 0.5 cm fraction from the anodes towards the cathodes. The individual fraction fragments were hereafter dissolved in 100 µl 1 M NaOH solution and counted for radioactivity.

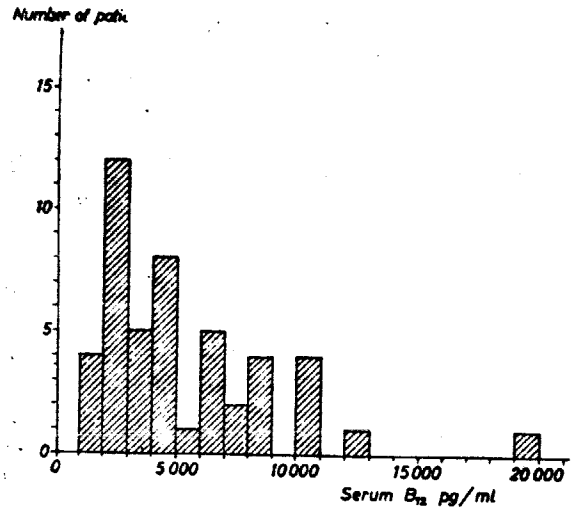

Fig. 1. Serum vitamin B_{12} in 47 patients with chronic myeloid leukemia.

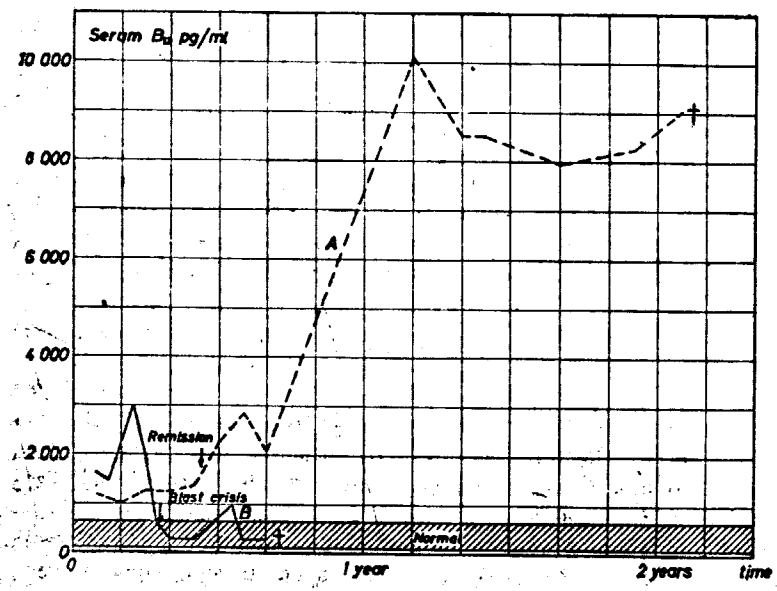

Fig. 2. Serum vitamin B_{12} in the course of chronic myeloid leukemia in one patient with elevated serum B_{12} in spite of remission (A) and in one patient with a fall in serum B_{12} in blast crisis (B).

the values of vitamin B_{12} in serum. Table I demonstrates the relative mobilities and the relative percentage distribution of protein fractions separated by means of agar gel micro-electrophoresis of concentrated extracts from normal individuals and from patients with chronic myeloid leukemia.

The table shows that in normal leucocytes six fractions appear, of which the fraction in the mobility interval from 0.23 to 0.38 is predominant, however, in case

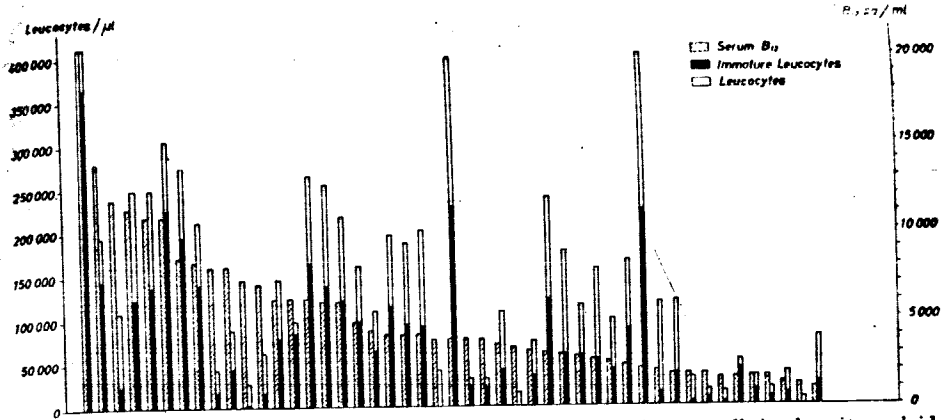

Fig. 3. Serum vitamin B_{12}, number of leucocytes and number of immature cells in chronic myeloid leukemia.

TABLE I

THE ELECTROPHORETIC DISTRIBUTION OF PROTEIN IN LEUCOCYTES FORM NORMAL INDIVIDUALS

Relative mobility (Mr) / *Percentage distribution*	*−0.3*	*0.06*	*0.23−0.38*	*0.47*	*1.07*	*1.22−1.44*
Case No. 1	0.0	13.5	13.5	46.0	27.0	0.0
Case No. 40	7.0	11.7	41.4	0.0	31.3	8.6
Case No. 131	0.0	12.1	45.0	0.0	33.6	9.3

Relative mobilities and percentage distribution of protein fractions separated by means of agar gel micro-electrophoresis. The fraction with a mobility of 1.07 is immunochemically identified as serum albumin.

No. 1, the predominant fraction is that possessing a relative electrophoretic mobility of 0.47. The fraction with a mobility of 1.07 immunochemically identified as serum albumin, makes up a rather constant percentage of the total protein of the leucocytes, namely between 27 and 33.6%.

In chronic myeloid leukemia, several more fractions are distinguishable by means of agar gel electrophoresis. Thus 11 fractions have maximally been separated. Of these no fraction with a distinct electrophoretic mobility seems to be predominant in all cases (Table II). The leukemic leucocyte seems to reveal several more fractions in the electrophoretic mobility interval from 0.06 to 0.89 than occurs in normal leucocytes. The fractions of this area are very distinct (Fig. 4). Immunoelectrophoresis (Fig. 5) of the leucocyte extract reveals in this area, prominent precipitation lines, possessing an electrophoretic mobility from α_1 to α_2 area. One of these takes up vitamin B_{12} as autoradiography reveals it to be radioactive (Fig. 6). In all cases both the normal leucocytes and the leucocytes from patients showed only this distinct vitamin B_{12} binding protein with an α-globulin mobility.

The combined immunodiffusion revealed the identity of the α-globulin of normal leucocytes with that of leukemic leucocytes.

After having made the autoradiographical pattern of immuno-electrophoresis slides, the dried agar was cut up into pieces, and the individual pieces were counted for radiography and the percentage distribution on the radioactivity was calculated

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TABLE II

THE ELECTROPHORETIC DISTRIBUTION OF PROTEIN IN LEUCOCYTES FROM PATIENTS WITH CHRONIC MYELOID LEUKEMIA

Relative mobility (Mr)	*−0.1*	*0.0–0.6*	*0.12–0.21*	*0.29–0.39*	*0.46–0.56*	*0.62*	*0.71*	*0.79–0.89*	*0.96*	*1.03–1.12*	*1.39*
Percentage distribution											
Case No. 27		20.5	18.1	19.7	17.3				18.9	5.5	
Case No. 59		7.0	14.6	9.2	13.5	10.8	24.3	7.0		11.4	2.2
Case No. 74		12.9	20.0	15.7	24.3			21.4		5.7	
Case No. 187	11.3	16.3	15.0	12.5				23.8	8.8		

Relative mobilities and percentage distribution of protein fractions separated by means of agar gel micro-electrophoresis.
The fraction with a mobility of 1.07 is immochemically identified as serum albumin.

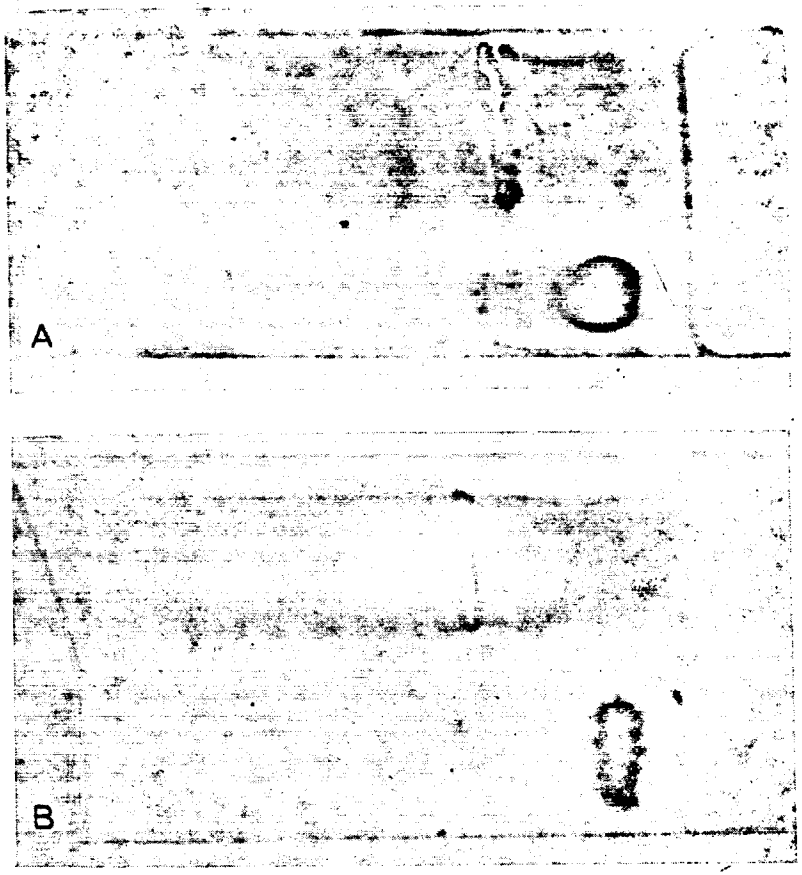

Fig. 4. Agar gel micro-electrophoresis of protein from normal leucocytes (A) and from leucocytes from patients with chronic myeloid leukemia (B).

(Table III). As the immuno-electrophoretic procedure involves the washing-out of the non-reacting immunological moieties of antigens and antibodies as well as elimination of the non-bound vitamin B_{12}, the vitamin B_{12} left in the immuno-precipitation lines may represent strongly bound vitamin.

From Table II it is obvious that the major part of the vitamin B_{12} is localised, in all normal leucocytes studied, in the α area. However, some of the radioactivity, namely 10.39 and 15% respectively, was localised in the γ, β_2 or β_1 areas. In the leukemic leucocytes there seems to be a trend to more binding in the γ and β areas than in the case of normal leucocytes (Table III). However, the material is small so that no definite conclusion can be placed upon the present findings. The binding in the β and γ areas may represent vitamin B_{12} bound to precipitin lines which are so weak that they are not shown up by the protein stains and not developed in the auto-radiographic pattern, or the vitamin B_{12} in this area is bound to colloids not precipitating with the antibodies used. The latter theory is supported by the more diffuse darkening of the X-ray film in this area.

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that the α-glob[illegible] with increased binding capacity in chronic myeloid leukemia, originates from [illegible]ucocytes[6,7]. The present communication deals with this question and should eluc[illegible]e a probable relationship between the total number of leucocytes in serum and the total vitamin B_{12} binding capacity.

MATERIALS AND METHODS

Material. The patient material consisted of 47 patients with chronic myeloid leukemia. The material was obtained from the Radium Station in Aarhus and from the Medical departments of the County Hospitals in the Copenhagen area.

Methods. Assay of vitamin B_{12} in serum was performed by means of Lactobacillus leishmanii[8,9]. With this assay, which was performed by Dumex Medical Company Ltd., Copenhagen, Denmark, a normal value of 455 pg/ml serum with a range from 125–720 pg/ml was found for 42 healthy adult control persons. Leucocytes were isolated from normal individuals and from patients with chronic myeloid leukemia[10,11]. 100 ml blood was mixed with 2000 I.U. heparin, and 20 ml 5% (w/v) dextran with a molecular weight of 250000. After 25 min standing the erythrocytes were sedimented and the supernatant plasma containing leucocytes was pipetted off. This plasma was centrifuged at 130 g in a Sorwall centrifuge (rotor head SS 34) at +4° for 4 min. This procedure was repeated twice. Hemolysis was induced by means of distilled water for 30 sec and thereafter an equal volume of 1.8% (w/v) NaCl was added. The procedure was repeated so that all erythrocytes were destroyed. The suspension of leucocytes in physiological saline was purified by centrifugation and resuspension in physiological saline three times. The leucocytes were isolated by centrifugation every time at 1000 *g* for 2 min in Sorwall centrifuge (Rotor head SS 34) at +4°.

In order to liberate the proteins of the leucocytes the suspension was deep-frozen to −80° during a period of 30 min. Hereafter the mixture was thawed and the suspension of membranes was further treated in a Potter-Elvehjem disintegrator at 40 rev./min for 2 min (0°). The protein liberated was concentrated to 3 g/100 ml by vacuum dialysis or by lyophilization.

Assay of total protein was performed by the method described by Lous *et al.*[12] and Lowry[13].

Production of antibodies against leucocyte extract was performed by the long-time immunisation schedule in rabbits. This schedule consisted of five times injection of 200 μl leucocyte extract i.m. in rabbits. The first injection was followed by a s.c. injection of 0.5 ml Freund's complete adjuvant. The individual injections were made at 3 weeks intervals and exactly 8 days after the last injection the animals were bled by heart puncture. The electrophorectic pattern of leucocyte extract was developed by the micro-electrophoretic technique of Wieme[14].

The electrophoresis was performed below light petrol ether (boiling point 40–60°) on microscope slides in 1 mm thick layer of 1% (w/v DIFCO Special Noble Agar) in 0.05 M sodium barbital solution at pH 8.6 and at 7 V/cm at 28°. 20 μl extract was applied in a 1 cm through placed in the middle of the electrophoretic slide. A standard solution of 2% (w/v) human albumin and dextran was used as electrophoretic markers for the 1.00 and 0.00 electrophoretic mobility. After electrophoretic separation for 24 min the slides were fixed for 10 min in a 5% aqueous glacial acetic acid, dried below filter paper at 40° and developed by staining in Amido Black solution. The electronic

[illegible]tern was semi-quantitatively evaluated by scanning [illegible] [illegible]ic in-[illegible]ator (Vitatron Ltd., Dieren, Holland).

The immunoelectrophoresis was performed as described by [illegible] [illegible] micro-scope slides in 1 mm thick 1% (w/v) agar solution (DIFCO Special Noble) in 0.05 M sodium barbital buffer at pH 8.6. 1.5 μl leucocyte extract was applied in the electrophoretic holes situated in the middle of the slide 1 cm apart from the intermediate antibody trough. The electrophoresis was performed at 24° at a field strength of about 4 V/cm. The electrophoretic separation was completed after $1^1/_2$ h. 80 μl antibody solution was applied in the central through, and the immuno-diffusion took place during 16 h at 24° in the electrophoretic chamber saturated with water. After immuno-diffusion the slides were washed in 0.9% sodium chloride solution for two days, desalted in distilled water for 1 h, dried, stained with Amido black. Assay of the binding capacity of leucocyte antigens for vitamin B_{12}: 90 μl leucocyte extracts were mixed with 10 ml Co^{58} labelled vitamin B_{12} (specific activity of 74 mμCi/μg Co^{58} (The Radio Chemical Centre, Amersham, England)) and incubated at 24° for 4 h prior to the immunoelectrophoretic separation. After electrophoretic separation, immuno diffusion and washing of the slides with physiological saline and distilled water the slides were dried but not stained. Thereafter, they were placed on ordinary X-ray film for autoradiography as suggested by Clausen and Munkner[16], and stored in a dark room for two months. By avoiding staining of the electrophoretic pattern, chemical catalysis of the X-ray film was avoided. After two months the slides were stained and the X-ray film developed. The autoradiographic patterns were compared with the protein pattern of the leucocytes.

The immuno-electrophoretic slides were then cut out in a 0.5 cm fraction from anodes towards the cathodes. The individual fragments were then dissolved in 100 μl 1 M NaOH solution and counted for radioactivity. The statistical evaluation of the data obtained, some of which were compared mutually by means of the correlation analysis[17], was performed on an Olivetti Programme No. 100 machine by means of the computer programmes No. 1:52[18].

RESULTS

In the 47 patients with chronic myeloid leukemia the serum vitamin B_{12} level was assayed to be between 1160–20,700 pg/ml with a mean value of 5292 pg/ml (the latter value was calculated on the basis of the first assayed value during hospitalization). The distribution of the vitamin B_{12} level is indicated in Fig. 1.

91% of all patients showed a serum vitamin B_{12} level above 2000 pg/ml.

In 28 patients the serum vitamin B_{12} was also assayed during the development of the disease. In 20 patients the values were increased during the whole progression of the disease, even in case of remission which occurred in 13 patients. Only in 8 patients the serum vitamin B_{12} values decreased during the development of the disease. In 7 cases this occurred in connection with a blast crisis, and only in one case the decrease occurred in relation to a remission of the chronic myeloid leukemia. In Fig. 2 the typical course for two patients is illustrated. The serum vitamin B_{12} values in all 47 patients are also related to the total leucocyte content of serum as well as to the number of immature cells of the peripheral blood (Fig. 3).

No significant correlation was found between the numbers of leucocytes and

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protein. However, it was shown that in leukemic leucocytes more radioactivity than normal was localized in the γ and β areas, where it was not possible to trace a precipitating line with radioactivity. This may be explained either by the presence of so small an amount of immuno-precipitate that it will not be possible to trace it, either by the staining method or by radiography, or by the fact that vitamin B_{12} in this area is bound to colloids not precipitated by specific antibodies.

Thus, in chronic meyloid leukemia as well as in normal leucocytes only one vitamin B_{12} binding protein with α mobility occurred supporting the idea that in chronic myeloid leukemia an increased number of leukemic leucocytes will liberate the vitamin B_{12} binding protein to the blood stream rather than liberating an abnormal leukemic protein with high serum vitamin B_{12} binding capacity.

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AN IMMUNOLOGICAL STUDY OF CATEGORIES OF GASTRITIS

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Summary Independent assessments of (1) the clinical data and findings on gastric microscopy and (2) the presence of gastric autoantibodies were made in a group of Australian patients with pernicious anæmia, idiopathic chronic atrophic gastritis, and post-gastrectomy gastritis. Intrinsic-factor antibodies, whether anæmia was present or absent, were invariably associated with profoundly impaired absorption of vitamin B_{12}. Parietal-cell antibodies were found in a high proportion of patients who gave evidence of either grossly or moderately impaired absorption of B_{12}, but they were uncommon in patients with normal absorption. Gastric antibodies were not found in patients with post-gastrectomy gastritis, whether absorption was impaired or not. Gastric autoantibodies were not present when the gastric mucosa was microscopically normal.

Introduction

ANTIBODIES to gastric antigens are present in the serum of most patients with pernicious anæmia and of some with chronic gastritis. The present investigation was undertaken to determine whether gastric antibodies had diagnostic significance and whether their presence permitted division of chronic gastritis into different categories. To avoid bias, the clinical status and the findings on microscopy of the stomach of patients with gastritis were assessed in Melbourne independently of the immunological studies, which were carried out in Palo Alto, California; the data were combined at the end.

Patients and Methods

Patients

21 patients with pernicious anæmia had achlorhydria, biopsy evidence of atrophic gastritis, a megaloblastic bone-marrow, serum-vitamin-B_{12} activity in those tested of less than 120 pg. per ml. and defective absorption of vitamin B_{12} as measured by the urinary excretion test (Schilling 1954). 35 patients had chronic atrophic gastritis without the hæmatological changes of vitamin-B_{12} deficiency. Serum-vitamin-B_{12} activity was measured in 32; and, in 29, the absorptive capacity of vitamin B_{12} was tested at least once.

Post-gastrectomy patients with biopsy-proven chronic gastritis had undergone operation, 13 on account of duodenal ulcer and 11 on account of gastric ulcer. Serum-vitamin-B_{12} activity was measured in all; and absorption of vitamin B_{12} was measured in 14 with low levels.

An unclassified group of 48 had normal findings on gastric biopsy: 41 were North Americans in good health; and 7 others (Australians) had diseases, including systemic lupus erythematosus (S.L.E.), which were unrelated to the gastrointestinal tract or endocrine system.

Serum-vitamin-B_{12} Activity

Euglena gracilis (Z strain) was used as the test organism, according to the method of Hutner et al. (1956) as modified by Anderson 1964).

Microscopy of Gastric Mucosa

Biopsies were carried out with the Wood or Rubin multipurpose tubes. The criteria of gastritis were glandular disorganisation and atrophy, and excess lymphocytes and plasma cells; these two indices were assessed independently on a 0 to +++ scale.

Gastric Antibodies

Gastric mucosa was obtained surgically from patients of blood-group O for the immunofluorescence tests. Cryostat sections were dried in air without fixation, since alcohol destroys the cytoplasmic antigen. The sections were treated with the test serum for thirty minutes, rinsed with Coons' buffered saline solution, and washed in buffer for ten minutes. Rabbit anti-human γ-globulin fluorescein conjugate was mixed in a 2:1 ratio with normal rabbit serum conjugated with rhodamine, the mixture being absorbed with an equal amount of rabbit liver powder. Sections were treated with the conjugate for thirty minutes, washed for ten minutes in Coons' saline solution, mounted in 50% buffered glycerol at pH 8·0, and examined by ultraviolet microscopy.

Hog-fundal mucosa was the source of antigen for complement fixation (C.F.) tests. The stomach was washed in buffered saline solution and the mucosa was separated from the muscle layer. The tissue was minced and incubated for fifty minutes with an equal volume of an 0·1% solution of ficin in 'Veronal'-saline buffer (V.S.B.); all procedures were carried out at 4°C. The tissue suspension was centrifuged in the cold at 7000 r.p.m. for ten minutes, and the sediment was washed with V.S.B. to

remove the ficin. The mucosa was disrupted in a Potter all-glass homogeniser with an equal volume of 30% sucrose, and filtered through muslin; mucus, cell debris, and nuclei were removed by centrifuging at 2000 r.p.m. for ten minutes. The supernatant was centrifuged at 40,000 r.p.m. in a 'Spinco' model L for fifty minutes; the deposit was evenly resuspended in V.S.B., lyophilised and stored at −20°C. Complement fixation was tested with this antigen according to a micro-modification of the method of Donnelly (1951) with 2 M.H.D. of complement.

Intrinsic-factor Antibodies

Lyophilised normal human gastric juice was collected from healthy volunteers after histamine stimulation; constant buffering in vivo was maintained with 0·07*M* phosphate buffer, pH 7·8, throughout a ninety-minute collection. All processing was carried out at 4°C. The juice was filtered through gauze and dialysed for twenty-four hours against five changes of a large volume of distilled water and was lyophilised. The samples were pooled on the basis of total colloid per unit volume of gastric juice. A fresh solution was made up for each test using isotonic saline solution buffered to pH 7·2 with phosphate. ^{60}Co-labelled vitamin B_{12} of specific activity 1 μC per μg. per ml. was used as a source of B_{12}. Serum-intrinsic-factor antibodies were assayed according to a modification of the method of Ardeman and Chanarin (1963) which detects the antibody by its ability to inhibit the binding of vitamin B_{12} by intrinsic factor (Fisher and Taylor 1965).

Thyroid Cytoplasmic Antibodies

Thyroid tissue obtained at surgery from thyrotoxic patients was prepared for immunofluorescence in the way described for gastric tissue.

Antinuclear Antibodies

Imprints of fresh calf-thymus were made on slides and fixed in acetone for ten minutes. The test sera were diluted 1:4 with phosphate buffer pH 7·0 and then applied to the imprint. The rest of the procedure was as already described.

TABLE I—MUCOSAL CHANGES

Diagnosis	No. of patients	Mucosal changes							
		Atrophy				Cellular infiltration			
		0	+	++	+++	0	+	++	+++
Pernicious anæmia	21	..	..	1	20	1	9	9	2
Gastritis	35	..	8	8	19	..	4	19	12
Post-gastrectomy (gastric ulcer) ..	11	1	1	1	8	..	2	4	5
Post-gastrectomy (duodenal ulcer)	13	..	3	4	6	..	2	8	3

Results

The degree of gastric atrophy and cellular infiltration in different groups of patients with gastritis, including patients with pernicious anæmia, is summarised in table I. There was a trend towards an inverse relation between them, which is not unexpected, since the number of lymphoid cells must to some extent depend on the total volume of mucosa. Statistical analysis of the data is hampered by the small numbers of patients and the dubious validity of ascribing equal values to the differences between 0, 1, 2, and 3 plus-scoring for both atrophy and infiltration. If this evaluation is permissible, however, there is a significantly greater degree of atrophy and a significantly lesser degree of cellular infiltration in pernicious anæmia than in the other types of gastritis. Thus, the difference between pernicious anæmia and chronic gastritis in group 2 (table I) is highly significant (P is $<0·005$) and the differences between pernicious anæmia and post-gastrectomy gastritis are significant for the gastric-ulcer cases ($P<0·04$) and the duodenal-ulcer cases ($P<0·005$) respectively. Differences in cellular infiltration between the groups are less striking, but there is less infiltration in pernicious anæmia than in chronic gastritis (group 2) or post-gastrectomy gastritis (groups 3 and 4), and all these differences just achieve the conventional limit of statistical significance ($P<0·05$).

TABLE II—PREVALENCE OF PARIETAL-CELL AND INTRINSIC-FACTOR ANTIBODIES

Diagnosis	No.	Gastric antibodies						Intrinsic-factor anti-body	
		Complement-fixation test*				Immuno-fluorescence			
		<4	4–8	16–32	64	−	+	−	+
Pernicious anæmia	21	4	7	9	1	2	19	10	11
Gastritis	35†	25	3	4	2	25	10	34	1
Post-gastrectomy (gastric ulcer) ..	11	11	..	..	..	11	..	11	..
Post-gastrectomy (duodenal ulcer)	13	13	..	..	..	13	..	13	..
Controls (normal mucosa) ..	48	48	..	..	..	48	..	48	..

* Reciprocal of titre. † 1 serum anti-complementary.

The prevalence of parietal-cell antibodies and intrinsic-factor antibodies is shown in table II, in which the groupings are as in table I. The results clearly confirm earlier observations that there is a significantly higher prevalence and, by complement fixation, a higher titre of gastric mucosal antibodies in pernicious anæmia than in any other group of patients. This table also shows the first data to be obtained from a group of controls in whom gastric biopsy has established (within the limits imposed by single or double biopsy) that no gastritis is present.

In pernicious anæmia 17 (81%) sera were positive by complement fixation, and 19 (90%) by immunofluorescence, and 11 (52%) sera contained intrinsic-factor antibodies. None of the 48 control sera was positive by any test.

In 35 patients with atrophic gastritis, 9 (27%) had sera positive for gastric antibodies by complement fixation and 10 (29%) by immunofluorescence, and 1 of these 10 sera was positive for intrinsic-factor antibodies. Of the 24 patients with post-gastrectomy gastritis, none had parietal-cell or intrinsic-factor antibodies.

The results of tests for thyroid acinar-cell antibodies are shown in table III. If weakly positive results are included, then 14 out of 21 (67%) pernicious anæmia sera were positive by immunofluorescence. Of the 35 gastritis patients, 11 (31%) had positive sera. Of the 24 post-gastrectomy patients, 12 (50%) had positive sera. Of the 48 controls, 7 (14%) were positive. Excluding weakly positive results, 12 of 21 (57%) pernicious anæmia sera, 7 of 35 (20%) gastritis sera, 6 of 24 (25%) post-gastrectomy sera, and 7 of 48 (14%) control sera were positive.

The prevalence of antinuclear factor is also shown in table III. Positive tests for antinuclear factor were obtained in 9 (42%) of the pernicious-anæmia group, 9 (25%) in the gastritis group, and 6 (25%) in the post-gastrectomy group. In the control group, 4 (9%) were positive, but all

TABLE III—THYROID MICROSOMAL ANTIBODIES BY IMMUNOFLUORESCENCE

Diagnosis	Total	Thyroid			Antinuclear factor
		W+	+	++	+
Pernicious anæmia ..	21	2	7	5	9
Gastritis	35	4	6	1	9
Postgastrectomy	24	6	6	..	6
Normal mucosa	48	..	6	1	4*

* Patients with systemic lupus erythematosus.

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4 positive sera were from patients known to have lupus erythematosus.

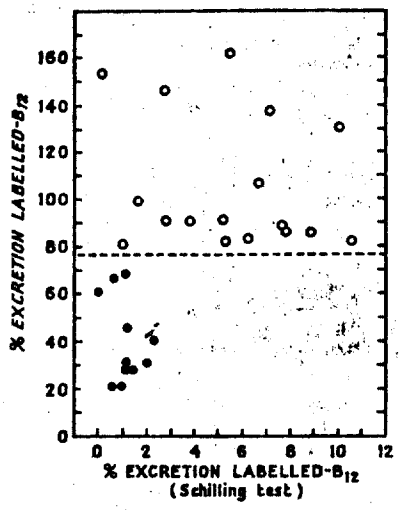
Discussion

Our findings indicate that neither parietal-cell antibodies nor intrinsic-factor antibodies occur if the gastric mucosa is normal on biopsy. The prevalence of parietal-cell antibodies in the largest, previously reported group of healthy people was 11%—5% under the age of sixty and 16% over the age of sixty (Taylor et al. 1962). This was an unselected population of 100 apparently healthy people, but none of these controls underwent biopsy and some might have had asymptomatic gastritis—a well-recognised abnormality (Joske et al. 1955). In a different approach to the correlation of circulating parietal-cell antibodies with the microscopic appearance of the gastric mucosa, Adams et al. (1964) and te Velde et al. (1964), showed that, in people whose sera were positive by gastric immunofluorescence, even single gastric biopsy specimens almost invariably disclosed evidence of chronic gastritis.

The high prevalence of gastric and thyroid antibodies in pernicious anæmia has been reported in many earlier observations; in our patients intrinsic-factor antibodies were present in 52%, which is in agreement with earlier figures of workers using a similar method (Ardeman and Chanarin 1963, Coghill et al. 1965, Fisher and Taylor 1965, Irvine 1965). Our high prevalence in pernicious anæmia of gastric microsomal antibodies by complement fixation (81%) may be due to the fact that hog, instead of human, gastric mucosa was used as antigen. The prevalence of parietal-cell antibodies in group 2, though significant, is somewhat less than that previously reported (Fisher and Taylor 1965). The present group, however, was drawn from a population geographically different from the earlier one and the prevalence of iron-deficiency anæmia may be lower. The present group also contains a higher proportion of males. Sex differences may be important in determining the formation of parietal-cell antibody in patients with atrophic gastritis (Coghill et al. 1965).

TABLE IV—CORRELATION OF VITAMIN-B_{12} ABSORPTION WITH SERUM-B_{12} ACTIVITY AND WITH GASTRIC ANTIBODIES IN PATIENTS WITH IMPAIRED B_{12} ABSORPTION

Group	Diagnosis on admission	Serum B_{12} (pg. per ml.)	Urinary excretion B_{12} (%)	Gastric antibodies		
				Immunofluorescence	Complement fixation test	Intrinsic factor
Urinary excretion test <5·0	Pernicious anæmia	100	0	+	<4	−
	,, ,,	40	0	+ +	4	+
	,, ,,	63	0	+ +	8	−
	Atrophic gastritis	50	0	+	4	−
	Pernicious anæmia	..	1	+	16	+ +
	,, ,,	53	1	+ +	32	+
	,, ,,	..	1	+	64	+ +
	Atrophic gastritis	120	1	−	<4	−
	Pernicious anæmia	40	1	+ +	8	+ +
	,, ,,	..	1	+	<4	+ +
	,, ,,	..	1	+	16	+
	,, ,,	62	1	+	8	+
	,, ,,	..	1	+	8	+ +
	,, ,,	..	2	+	32	−
	,, ,,	40	2	+	32	+ +
	Atrophic gastritis	20	2	+ +	16	+
	Pernicious anæmia	110	3	+ +	8	−
	,, ,,	..	3	+ +	16	−
	,, ,,	..	4	+	32	−
Urinary excretion test >5·0	Pernicious anæmia	..	5	−	<4	−
	,, ,,	80	5	−	<4	−
	Atrophic gastritis	70	5	+	8	−
	,, ,,	56	6	+	<4	−
	,, ,,	160	6	−	<4	−
	,, ,,	..	8	+	64	−
	,, ,,	240	8	−	<4	−
	,, ,,	130	8	+	32	−
	,, ,,	20	8	+ +	16	−
	Pernicious anæmia	40	9	+	16	−
	Atrophic gastritis	40	10	+ +	32	−
	Pernicious anæmia	100	10	+	32	−



Correlation of intrinsic-factor antibodies and Schilling test.

% retention of labelled B_{12} is the percentage of B_{12} retained compared with the constant standard of normal pooled serum (Fisher and Taylor 1965).

Broken line, 2 standard deviations below normal mean of 95% intrinsic-factor antibodies present when % retention ≤78%. O negative for antibody; ● positive for antibody.

Our data permit examination of a question which has excited much interest—namely, what are the criteria by which a patient with chronic atrophic gastritis may be judged to have pernicious anæmia? Atrophic gastritis is an invariable finding in the adult addisonian type of pernicious anæmia. The degree of cellular infiltration varies, but considerable atrophy seems to be invariable. Current techniques of microscopy, however, cannot differentiate patients with chronic gastritis into those with pernicious anæmia and those without (Shiner and Doniach 1957). If a histochemical means of identifying Castle's intrinsic factor became available, then differentiation might be possible. Meanwhile, pernicious anæmia can be confidently diagnosed if the patient has a megaloblastic anæmia, a serum-vitamin-B_{12} activity in the range for pernicious anæmia (below 100 pg. per ml.), a histamine-fast achlorhydria with atrophic gastritis, impaired vitamin-B_{12} absorption in the range for pernicious anæmia and with absorption being restored to normal with intrinsic factor, and absence of intrinsic factor in gastric juice. Recent developments suggest that the diagnosis of pernicious anæmia may be strengthened by the presence in serum and in gastric juice of parietal-cell microsomal antibodies and intrinsic-factor antibodies, particularly the latter (Fisher et al. 1965, 1966).

If patients with pernicious anæmia could be separated from patients with chronic gastritis displaying some, but not all, of the features enumerated, our understanding of pernicious anæmia would be advanced. Faber's (1935) hypothesis that gastritis is a progressive disease, ranging from superficial through atrophic gastritis to the final stage of pernicious anæmia could then be proved or disproved. Many of the current diagnostic tests, however, yield results which overlap considerably. Even tests of vitamin-B_{12} absorption, which are usually regarded as conclusive in the diagnosis of pernicious anæmia, may

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overlap considerably in the case of patients with frank pernicious anæmia, gastritis, and in healthy controls (Doscherholmen 1965).

Table IV lists all of the patients in whom urinary excretion tests (U.E.T.) were carried out in order of their increasing capacity to absorb vitamin B_{12}. The three groups are characterised by a U.E.T. of 0 to 5%, 5 to 10%, and above 11%.

In the group of 19 with a Schilling test of 0 to 5%, 3 (classed as chronic gastritis) had no hæmatological evidence of vitamin-B_{12} deficiency, although all had abnormally low serum-vitamin-B_{12} activity. Parietal-cell antibodies were present in 18, and the 10 patients with intrinsic-factor antibodies were found in this group. In the accompanying figure the titre of intrinsic-factor antibody is plotted against the B_{12} urinary excretion values in patients with urinary excretion of 10% or less; all patients with circulating intrinsic-factor antibodies had grossly impaired B_{12} absorption. Some patients (5 out of 16) with the same degree of malabsorption, however, had no detectable intrinsic factor antibodies. This may have been due to lack of sensitivity of the best available method, but we suggest that there may be three different types of pernicious anæmia: first, a type with severely impaired vitamin-B_{12} absorption and no intrinsic-factor antibodies; secondly, a type with as severe an impairment of absorption and with intrinsic-factor antibodies; and thirdly, a type with somewhat less severely impaired B_{12} absorption and without intrinsic-factor antibodies. One may speculate that vitamin-B_{12} malabsorption would be less pronounced in the second type if intrinsic-factor antibodies were not present. Both type 2 and type 3 may retain the capacity to produce diminished amounts of intrinsic factor, but in type 2 the available intrinsic factor might be neutralised by antibody. The finding of intrinsic-factor antibodies in gastric juice of some pernicious-anæmia patients (Fisher et al. 1966, Schade et al. 1966) lends support to this speculation.

The 3 patients in group 1 (table IV) diagnosed as having atrophic gastritis seem to have the essential lesion of pernicious anæmia—namely, a failure to absorb vitamin B_{12} on the basis of gastric disease with resulting low serum-vitamin-B_{12} activities. One way of explaining why hæmatological or neurological disease has not developed in them is that their metabolic requirements for vitamin B_{12} are different from others. This is in accord with the finding that in some people vitamin-B_{12} deficiency may result in severe hæmatological disorders without neurological disease, and vice versa. Another possible explanation is that variation in intake of folic acid may have an important role in determining whether hæmatological changes occur or not. These 3 patients in group 1 may justifiably be classed as having pernicious anæmia or pre-pernicious anæmia, but this implies that if they had been left untreated, hæmatological or neurological changes might have ensued. There is clearly a need to define by general agreement the term pernicious anæmia, with particular reference to impaired absorption of vitamin B_{12} and to variation in hæmatological, neurological, and other metabolic requirements of vitamin B_{12}.

The second group of 12 had U.E.T. results between 5% and 10%, which is still within the range usually regarded as abnormal. As with group 1, there is fairly good agreement between the absorption defect and the serum-B_{12} activity, bearing in mind that the microbiological serum assay varies considerably and that nutritional deficiency

TABLE V—CORRELATION OF VITAMIN-B_{12} ABSORPTION WITH SERUM-VITAMIN-B_{12} ACTIVITY AND WITH GASTRIC ANTIBODIES IN PATIENTS WITH NORMAL B_{12} ABSORPTION

Serum B_{12} (pg. per ml.)	Urinary excretion B_{12} (%)	Gastric antibodies		
		Immuno-fluorescence	Complement-fixation test	Intrinsic factor
100	11	–	<4	–
160	11	–	<4	–
130	11	–	<4	–
180	11	+	64	–
140	12	–	<4	–
140	13	–	<4	–
210	14	–	<4	–
140	15	–	<4	–
130	15	–	<4	–
114	16	–	<4	–
400	16	–	<4	–
130	17	–	<4	–
180	17	–	<4	–
240	18	–	<4	–
180	19	–	<4	–
210	19	–	<4	–
180	19	–	<4	–

and even a high dietary intake of vitamin B_{12} could influence serum levels. The same comments could be made about the patients in group 2 classed clinically and hæmatologically as atrophic gastritis without pernicious anæmia as about the similar 3 patients in group 1. The other indices are less impressive, however, and do not so strongly suggest that these subjects, in fact, have pernicious anæmia without yet having signs of vitamin-B_{12} deficiency. Nevertheless, some of the serum-vitamin-B_{12} levels are well within the range for pernicious anæmia, and the U.E.T. results are within the usually accepted range; moreover, 67% have parietal-cell antibodies, and the patients classed as pernicious anæmia are no more positive immunologically than the patients with atrophic gastritis. Arguably therefore all the subjects in group 2 have the gastric lesion of pernicious anæmia. Is it significant that none has intrinsic-factor antibodies?

A greater disparity between the U.E.T. and serum-vitamin-B_{12} levels is apparent in the 17 subjects (table V) than is evident in groups 1 and 2 (table IV) in which impairment of absorption is greater. Here, absorption is in the normal range, and the serum-vitamin-B_{12} activities are "low normal"—i.e., 150–250 pg. per ml. in 8, and in that "no-man's-land" of doubtful significance between 100–150 pg. per ml. in 8, but in 3 of these the U.E.T. (> 15%) is certainly normal. Possibly some of these subjects had mild dietary vitamin-B_{12} deficiency. The number of positive tests to gastric antigens is almost nil, there being only 1 with parietal-cell antibodies. None had intrinsic-factor antibodies. These patients almost certainly have not got the gastric lesion of pernicious anæmia, but only a long period of observation will clarify this issue.

Finally, the results for the post-gastrectomy patients, who underwent operation on account of gastric and duodenal ulcer, disclosed, despite a degree of gastritis comparable with that of group 2 in table I, an absence of immunological response (table II). In this respect these patients resemble those listed in table V; several of them had serum-vitamin-B_{12} levels and vitamin-B_{12} absorption well in the pernicious-anæmia range. This suggests that B_{12} deficiency is not a factor in the immunological response.

It has been argued that the gastric changes in pernicious anæmia are in some way related to an immune response, though no evidence has been adduced to establish the nature or site of this response, nor whether it plays a causal

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role in the development of the gastric lesion. The prevalence of circulating gastric antibodies is very high in pernicious anæmia, but in each reported series a few patients with this disease are recorded in whom no antibodies were detectable. Taylor et al. (1962) showed that the number of serologically positive patients was lower in the older age-group. They postulated that this was a reflection of total destruction of mucosal elements and removal of stimulating antigen, but an analysis of data for both parietal-cell antibodies and intrinsic-factor antibodies in terms of duration of the disease revealed no confirmatory correlation (Taylor et al. 1962). Hence these serologically negative cases are unlikely to be accounted for by the disease being " burnt out ", as has been suggested for those serologically negative cases of myxœdema which are presumed to be an end result of autoimmune thyroiditis.

From the present investigations there emerges an apparently direct correlation between the degree of depression of gastric function, as judged by absorption of vitamin B_{12}, and the prevalence of antibodies, but the underlying nature of this relation is not clear. Also, the microscopical findings in the gastric mucosa of patients with strong evidence of autoimmune response do not differ greatly from those without evidence of disturbed immune response. In the post-gastrectomy patients this is particularly striking, but it is also evident in the chronic-gastritis patients with little or no evidence of impaired production of intrinsic factor. Thus, not all inflammatory lesions associated with some degree of atrophy have an immune basis; or, to express this in another way, considerable damage of the gastric mucosa does not always result in an autoimmune response. Any simple concept, such as that release of previously immunologically sequestered antigenic material stimulates an antibody response, appears untenable. Increased understanding may follow a search for qualitative differences in the local inflammatory reaction.

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Folia primat. 4: 432–437 (1966)

Department of Anatomy, University of Birmingham

THE RELATIONSHIP BETWEEN GROWTH AND THE ADMINISTRATION OF CYANOCOBALAMIN IN THE RHESUS MONKEY

By R. M. FLINN and C. E. OXNARD

Introduction

Many studies have shown that a deficiency of vitamin B_{12} leads to gross abnormalities of growth and development in most animals (for review see SMITH [1965]). After birth the deficiency is often manifested as a decrease in rate of growth, and although vitamin B_{12} has little effect on growth when the food intake of the animal is artificially controlled (BOSSHARDT, PAUL and BARNES [1950]; BLACK and BRATZLER [1952]), it is nevertheless clear that the vitamin is necessary for the optimal growth of many animals maintained on otherwise normal diets.

The possibility therefore, that rhesus monkeys born in captivity might have lower rates of growth than might otherwise have been expected, thus follows (a) from the finding that they have smaller amounts of vitamin B_{12} in the serum (20–70 μμg/ml) as compared with those of recently captive animals (110–680 μμg/ml; KROHN, OXNARD and CHALMERS [1963]), and (b) more particularly from the report that, while the newborn of animals captive for long periods have low levels only slightly in excess of those of their own deficient mothers (both less than 100 μμg/ml of serum), the near-term foetuses or newborn of recently captive rhesus monkeys have amounts of the vitamin in the serum many times higher than those of their own mothers (mean of mothers—122 μμg/ml, mean of corresponding foetuses and infants—579 μμg/ml; OXNARD [1964]). The possibility is strengthened by the suspicion that infant rhesus monkeys born of mothers which have been captive for long periods appear to show spurts of growth

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after the administration of cyanocobalamin parenterally (OXNARD [1964]).

Because vitamin B_{12} deficiency had already been suggested as having a number of other effects on the health of the captive rhesus monkey (OXNARD [1965]), it was judged necessary, in the interests of having experimental animals as normal as possible, to treat most of the animals in the Birmingham monkey colony. This lead to a number of infant rhesus monkeys receiving the vitamin and thus made possible a retrospective analysis of the effect of treatment on the changes of body weight with age.

Materials and Methods

Experimental Animals

The animals used in this investigation were born in the Birmingham monkey colony between 1946 and 1963. As far as could be ascertained from records, all were normal—those animals which had been used in other experiments during infancy or in which there was any history of illness having been rejected.

Male and female infants born since 1961 were given, after weaning, intramuscular injections of cyanocobalamin (500 μg) at approximately monthly intervals. Determinations of the amounts of the vitamin in the serum in a sample of the animals after treatment confirmed that it was present in the circulation in large amounts. Records of the weight and age of the animals from birth to before the pubertal growth spurt were examined.

Control data for males and females were obtained from a survey of the records of monkeys born in the colony over the last twenty years, mainly from 1946 to 1954. These animals had received essentially the same diet as those recently born.

Statistical Treatment of Data

The regression of weight on age was calculated for each animal before and after treatment with cyanocobalamin. For the control animals an arbitrary separation into early and late growth was made, the point of division being at an age similar to that of the treated animals when they first received injections of the vitamin.

A covariance analysis was performed between the resulting eight groups of regressions in order to assess differences between early and late growth in the treated and untreated animals of each sex sub-group. Tests of significance were also carried out for the degree of change in regression in each individual between treated and control animals within the sex sub-groups.

Results

The examination of the data using analysis of covariance shows that the change in the regression coefficient of late growth as com-

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pared with that of early growth within any one group of animals is not significant. This is because of the small numbers of animals and wide spread of individual regressions within each group. The mean square for differences among regressions is greater than that attributable to the experimental error in five of the eight groups.

The examination of the data longitudinally, however, reveals that there are significant variations (t-test, $P = 0.005$) between the regressions of early and late growth of animals in the different

Table I. Changes in growth in individual rhesus monkeys treated with vitamin B_{12} contrasted with untreated monkeys.

Monkey	Regression of weight on age: Early growth	Regression of weight on age: Late growth	Ratio of early to late growth	Difference of early and late growth
Untreated females (6 individuals)	4.701	3.743	1.256	+0.958
	4.939	2.639	1.871	+2.300
	4.389	3.868	1.135	+0.521
	5.102	4.290	1.189	+0.812
	5.933	4.470	1.327	+1.463
	4.875	2.873	1.697	+2.002
Treated females (5 individuals)	3.757	4.404	0.853	—0.647
	3.240	4.456	0.727	—1.216
	3.313	5.510	0.601	—2.197
	5.708	5.121	1.114	+0.587
	4.453	4.768	0.934	—0.315
Untreated males (6 individuals)	4.237	1.986	2.133	+2.251
	4.929	2.663	1.851	+2.266
	4.038	4.115	0.981	—0.077
	3.293	1.853	1.777	+1.440
	4.030	2.775	1.452	+1.255
	3.545	2.116	1.675	+1.429
Treated males (4 individuals)	4.289	7.768	0.552	—2.479
	6.338	7.609	0.833	—1.271
	5.514	5.268	1.047	+0.246
	5.848	6.535	0.895	—0.687

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groups (Table I). These variations, as measured by the ratio and difference of late and early growth, show that the rate of growth of animals of each sex is greater after treatment with vitamin B_{12}.

These changes in growth are demonstrated by the appearances of a sample of the regressions of growth on age (Fig. 1). In this graph are displayed the regressions for the two animals of each sex subgroup which showed the least and greatest change respectively between growth before and after the administration of vitamin B_{12}. Super-

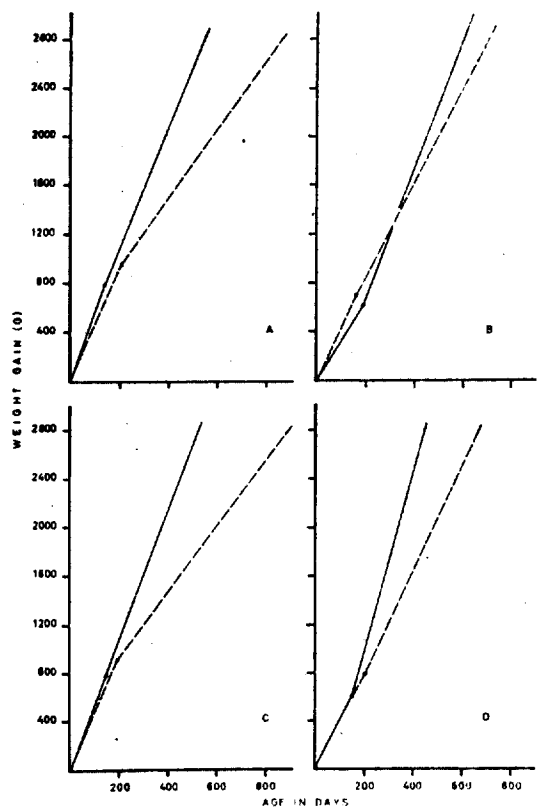

Fig. 1. Comparison of growth regressions in the rhesus monkey. Females showing maximum decrease (A) and maximum increase (B) in late growth as compared with early growth. Males showing maximum decrease (C) and maximum increase (D) in late growth as compared with early growth. - - - - - - - untreated animals. ——— = animals given vitamin B_{12} during period of late growth.

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imposed on this are the equivalent regressions for early and late growth in the appropriate control animals. The association of administration of vitamin B_{12} with the change of direction of growth is obvious in each case.

Discussion

The findings of this study are subject to two qualifications. Firstly, in order to obtain control data, it has been necessary to examine the records of animals which have been reared in the Birmingham colony at different times during many years. Although diets have remained more or less standard over this period, many other environmental factors must have varied and the adequacy of such controls may well be challenged.

Secondly, with the number of animals that were available, the variation between individual growth curves is so great as to obscure, when attempts are made to compare average growth rates, such changes as there may be present. Thus although the two groups of females have somewhat similar early growth rates, three of the four treated males already had considerably higher rates of growth before the administration of the vitamin than had the untreated group at the appropriate age. It is interesting to note, however, that the mothers of these three animals were the only recently captive mothers in the study and presumably therefore were in a more normal state of vitamin B_{12} nutrition during pregnancy (OXNARD [1964]).

Because, however, the findings are fairly clear cut despite the above qualifications and because planned growth studies on Primates are more difficult to carry out than is the case with small laboratory animals, it has been felt worthwhile to report such information as is available.

Thus the longitudinal comparison of the data relating to the change in growth rate within individuals shows clearly that treatment with vitamin B_{12} is associated with an increased rate of growth. All animals save one in the control group had lower rates of growth in late infancy compared to early life. In the treated monkeys, on the other hand, only two animals had rates which were lower after treatment, and those to only a fractional degree. In general, growth rates in late infancy after treatment show considerably less variation and are of the order of 50 to 200% greater than those in the same period in the untreated rhesus monkey.

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Such changes in growth are not only statistically significant but are large enough to have biological significance in the rearing of healthy rhesus monkeys. As it has also been shown that low levels of vitamin B_{12} in the serum have been found in some other members of the Anthropoidea after long captivity (OXNARD [1966]), it may be that for optimum growth all apes and monkeys ought to receive diets incorporating animal food or supplements to the diet of vitamin B_{12}.

Summary

A survey of the body weights of rhesus monkeys born and reared in the Birmingham colony has shown that there are significant increases in growth in infants treated with cyanocobalamin after weaning, as compared with the growth patterns in untreated monkeys.

ACKNOWLEDGEMENTS

We are indebted to Professor P. L. KROHN, F.R.S., for helpful discussion and criticism. The analysis of the data was aided by a grant from the Agricultural Research Council of Great Britain. The text figure was prepared by Mr. W. J. PARDOE.

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Food and Drug Administration Undated

B_{12} Specifications from Supplier

Food and Drug Administration, U.S. Department of Health, Education and Welfare

Food Protection Committee 1972

Comprehensive GRAS Survey

National Academy of Sciences, National Research Council, Washington, D.C.

Food Protection Committee, Food and Nutrition Board

Chemicals Used in Food Processing

National Academy of Sciences, National Research Council, Washington, D.C.
Publication 1274

Lancet 1:896

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Preliminary Communication

HYDROXOCOBALAMIN IN THE TREATMENT OF LEBER'S HEREDITARY OPTIC ATROPHY

Summary Two patients with Leber's hereditary optic atrophy had been treated unsuccessfully with cyanocobalamin. The discovery that hydroxocobalamin is superior to cyanocobalamin in the treatment of tobacco amblyopia prompted a trial of this compound in the two patients. The dose was 1 mg. twice weekly in one, and 1 mg. weekly in the other. Visual acuity in both eyes improved, though in one case this improvement might have been fortuitous.

INTRODUCTION

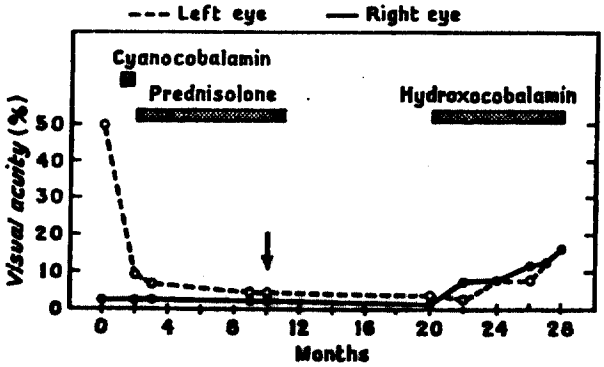
Leber's hereditary optic atrophy may result from a genetically determined sensitivity to certain constituents of tobacco smoke, perhaps cyanide or cyanogens,[1] and a defect in the detoxication of cyanide to thiocyanate has been suggested.[2] Chronic cyanide toxicity has been postulated in other conditions, for example, in the retrobulbar neuritis of vitamin-B_{12} deficiency[3] and in tobacco amblyopia,[4] and a favourable response of tobacco amblyopia to treatment with parenteral hydroxocobalamin even when smoking, the presumed source of cyanide, is continued has been described.[5]

We have treated two cases of Leber's hereditary optic atrophy with hydroxocobalamin.

CASE-REPORTS

Case 1

This man, aged 35, was seen at the Western Infirmary, Glasgow, with a year's history of numbness and paræsthesiæ affecting the right side of the body and coincident blurring of vision of the right eye. Similar blurring of vision of the left eye had been present for 1 month. He smoked 40 cigarettes daily and drank several pints of stout per day. On examination the visual acuity of the right eye was 2/60 and that of the left 6/12, with a dense central scotoma in the right field of vision and a smaller centrocæcal scotoma, more striking to a red target, in the left. The right optic disc was pale and the left œdematous. The right plantar response was extensor but there were no other abnormal neurological signs.



Case 1: response of the visual acuity to treatment.
The arrow indicates the time at which the patient was registered blind.

During the next 4 weeks the vision of the left eye deteriorated to 1/60 with an increase in the size of the central scotoma. There was striking bilateral optic atrophy. Leber's hereditary optic atrophy was diagnosed when it was discovered that his maternal uncle had been diagnosed as a case of Leber's hereditary optic atrophy in 1937, at the age of 36. The patient was registered as blind. The serum-vitamin-B_{12} level was 150 pg. per ml. (*Euglena gracilis* method) but other laboratory investigations were not significant.

He was treated first with cyanocobalamin 1 mg. intramuscularly on alternate days for 2 weeks and then with prednisolone commencing with 40 mg. daily reducing to 5 mg. daily over 2 months, and thereafter at this dosage for 8 months longer, but the visual acuity remained unchanged.

Some 2 years later, when the superiority of hydroxocobalamin over cyanocobalamin in the treatment of tobacco amblyopia was realised, the patient was treated with parenteral hydroxocobalamin 1 mg. daily for 2 weeks and thereafter twice weekly. Before starting this treatment the acuity of the right eye was 2/60 and of the left 1/60.

After 3 months the visual acuity had improved to 3/60 in each eye and after 6 months it was 6/60 in the right eye and 6/36 in the left. The patient could now read large print and was no longer considered registrable as blind. The progress of the visual defect is shown graphically in the figure.

Case 2

The condition of this 55-year-old farm bailiff has been outlined elsewhere[1] and will be only briefly summarised here. Rapid visual failure with a large central scotoma affected first the right eye and then, after 3 months, the left, in 1961. The condition was first diagnosed as tobacco amblyopia, but the family history of affected males related through the female line and the degree of visual impairment, the nature of the scotomata, and the degree of optic atrophy, made a diagnosis of Leber's hereditary optic atrophy inescapable. He smoked 3 oz. (80 g.) of tobacco weekly. His serum-vitamin-B_{12} level was 225 pg. per ml. (*Lactobacillus leichmanii* method).

He was advised to stop smoking completely, and treatment with cyanocobalamin 1 mg. intramuscularly twice weekly was continued for 5 months without benefit. By this time he could only count fingers at 25 cm. Treatment with hydroxocobalamin 1 mg. intramuscularly weekly was then substituted and over the next 18 months acuity slowly improved to 6/9 in the right eye and 6/60 in the left. Colour vision was restored but the left optic disc remained atrophic.

DISCUSSION

A certain degree of spontaneous improvement of visual acuity is not unusual in Leber's hereditary optic atrophy after the first sudden deterioration, but this did not happen in these two patients. Occasionally improvement is considerable[6] and in certain families visual impairment is unusually slight.[7] One must concede therefore that the improvement in case 2 may have been fortuitous and related neither to cessation of smoking nor to treatment with hydroxocobalamin.

In case 1, however, the timing of improvement after hydroxocobalamin which has a strong affinity for cyanide, in relation to the duration of visual impairment is quite exceptional and unlikely to be fortuitous, and it supports the hypothesis concerning the role of smoking in this condition.[1] Although a certain degree of optic atrophy persists in these patients and presumably certain neuroretinal elements are destroyed, the degree of improvement suggests that either remyelination of critically important fibres has occurred, or that certain neuroretinal tissues were malfunctioning rather than dead. If this is so, it may yet be possible to improve acuity in patients with long-established Leber's hereditary optic atrophy, even

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although some irreversible changes may have developed. The greatest promise must lie in treating those patients whose visual symptoms are recent, and in identifying those at risk before visual symptoms are established.

We are carrying out a more rigorous trial of this treatment in patients with Leber's hereditary optic atrophy.

ADDENDUM

The progress of one of Prof. R. W. Gilliatt's patients with Leber's disease has been reviewed. Treatment of this 18-year-old male with weekly injections of 1 mg. hydroxocobalamin was commenced 6 months after the onset of visual failure when visual acuity was 6/24 in the right eye and reduced to finger-counting with the left, earlier deterioration having occurred despite corticotrophin injections. The patient was advised to stop smoking when vitamin B_{12} was started and now, 2 years later, acuity is 6/18 (right) and 6/36 (left).

We thank Dr. Michael Kremer for permission to describe his patient from the National Hospital for Nervous Diseases.

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Effects of Vitamin B_{12} and Methionine on Excretion of Formiminoglutamic Acid by the Chick.* (26735)

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Young rats fed a diet deficient in Vit. B_{12} were found by Silverman and Pitney(1) to excrete large amounts of formiminoglutamic acid (FGA), a metabolite of histidine that normally is excreted in negligible amounts in urine. They found that supplements of either Vit. B_{12} or methionine caused a drop in FGA excretion to low or zero levels. This Vit. B_{12}-methionine relationship in the rat was closely analogous to that observed by Fox *et al.*(2,3) in young chicks; *i.e.*, growth was poor in the absence of Vit. B_{12} but good upon supplementation of the diet with either the vitamin or methionine. The diets used in both the rat and the chick studies contained high levels of fat and were borderline in the sulfur-containing amino acids. FGA excretion pattern of the Vit. B_{12} deficient chick was investigated because it seemed that this parameter of deficiency could be useful in elucidating some of the interrelationships between Vit. B_{12}, methionine, and fat. It was found that the Vit. B_{12} deficient chicks excrete large amounts of FGA and that this excretion rate is markedly reduced by methionine.

* Part of these data were presented at Fifth International Congress on Nutrition, Sept. 1-7, 1960, Washington, D. C.

TABLE I. Effect of Vit. B_{12} Deficiency upon Excretion of Formiminoglutamic Acid.*

Vit. B_{12}, mg/kg diet	Body wt ± S.E., g	Histidine intake ± S.E., mmole/day	μmoles FGA ± S.E. Per day	Per mmole histidine
0	114 ± 3.3	1.1 ± .07	113 ± 13.9	108 ± 14.5
.1	215 ± 8.8	2.0 ± .11	20 ± 5.0	11 ± 2.7

* Avg of values from 3 exp., total of 21 chicks for each treatment, 22 days of age.

Methods. Day-old female New Hampshire chicks were placed in electrically heated batteries with screen wire floors. They were fed diet C62(4), a purified soybean protein diet that contained 24% of fat. Vit. B_{12} was omitted from the diet except as indicated in Table I. After 3-5 wks, the chicks were placed in individual cages and the excreta were collected and assayed for FGA as previously described(4,5). The number of chicks per experimental group varied between 5 and 8. During the collection periods, L-histidine HCl was added to the diet at a level of 1% to accentuate the differences in FGA excretion. Supplements of methionine and related compounds were incorporated into the diet for 24 hr intrevals as indicated in Tables II and III. In the experiments reported in Table IV, chicks were raised on a Vit. B_{12} deficient diet exactly like C62 except that fat content was lowered to 4%; glucose was added to compensate for the weight of fat (200 g/kg of diet) that was omitted. FGA excretion was determined while the chicks received the low level of dietary fat and also during the 2 days following an increase in dietary fat to 24%. In some experiments food intake was restricted on certain days. When this was done, the chicks received one-third of the day's ration at the beginning of the day and the remaining two-thirds 8 hr later.

In addition to colorimetric assays for FGA many extracts of excreta were also subjected to paper chromatography and paper electrophoresis. Components of the extract were separated in duplicate on Whatman 3MM paper by ascending chromatography for 16 hr. The developing solvent was the butanol phase which separated after mixing n-butanol 40%, glacial acetic acid 10%, and water 50% by volume. The strips were dried and one strip for each sample was exposed to the atmosphere over ammonium hydroxide for 1 hr to hydrolyze the formimino group from

TABLE II. Effect of Methionine Supplementation for One Day upon Excretion of FGA by Vit. B_{12} Deficient Chicks.*

Day	DL-methionine, % of diet	Body wt, g	Histidine intake, mmole/day	μmoles FGA Per day	Per mmole histidine
1	0	203	2.0	103	52
2	0	212	1.9	111	58
3	1	231	2.6	14	5
4	0	232	1.8	270	150
5	0	239	1.9	121	64

* Mean values for 15 chicks from 2 experiments. Chicks were 31 days of age on day 1.

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TABLE III. Effect of Compounds Related to Methionine upon Excretion of FGA by Vit. B_{12} Deficient Chicks.* (μmoles FGA/day/mmole histidine intake.)

Day	Supplement†	DL-methionine‡	DL-homocysteine thiolactone HCl	DL-homocystine	L-cystine	Choline chloride	Betaine
1	0	67	113	39	35	68	46
2	0	72	111	34	51	45	59
3	+	35	111	39	50	48	72
4	0	211	113	68	66	74	60
5	0	120	103	46	43	44	48
No. of chicks		5	7	13	5	13	12

* When more than 7 chicks received a given supplement, the results are the avg of 2 experiments; ages ranged between 21 and 31 days.

† The methionine supplement (Table II) was incorporated at a level of 1% of the diet; all supplements in this table were equimolar or equivalent in methyls (choline and betaine) to methionine.

‡ Food intake of each chick was limited on day of methionine supplementation to amount of diet consumed the previous day.

the FGA. Excess ammonia was dissipated from the strips by forced air at room temperature for ½ hr. All strips were then dipped in a solution of 0.2% ninhydrin in acetone. FGA does not stain with ninhydrin whereas the glutamic acid formed during hydrolysis stains blue. Other pairs of strips were sprayed with the same alkaline ferricyanide-nitroprusside reagent and sodium borate solution as used for the colorimetric assay(5). These reagents form a pink color with compounds having a formimino group. Electrophoresis was carried out with the pyridine-acetate buffer of Knowles(6) in Spinco Model R migration chambers at 400 volts for two and one-half hr. A sample stripper was used to apply 0.01 ml of sample across each strip of 3 cm wide Whatman 3MM paper. The same volume of a standard solution containing 1 μmole FGA/ml was used as a control. Following electrophoresis, the strips were dried and cut in half longitudinally. One of each pair was exposed to ammonia vapor and then both strips were stained in the same manner as described above.

Results. The Vit. B_{12} deficient chicks grew slowly and excreted more than 5 times as much FGA as chicks in the control group receiving Vit. B_{12} (Table I). This difference was highly significant ($P<0.01$) when total mean daily excretions were compared as well as when FGA values were related to dietary histidine intake. Injection of Vit. B_{12} deficient chicks with 1 γ Vit. B_{12}/day has been reported to cause FGA excretion to decline to minimal levels after 2 days(4). The same effect has been observed when the diet of the Vit. B_{12} deficient chicks was supplemented with 0.1 mg Vit. B_{12}/kg of diet (unpublished data).

When Vit. B_{12} deficient chicks were given a DL-methionine supplement (incorporated in the diet at a level of 1%) for 24 hr, there was an immediate and marked drop in FGA excretion (Table II). On the day following methionine supplementation, FGA excretion rose to a level which was more than double the daily excretion during the pre-supplementation period. Then the amount of FGA dropped to the original level on the fifth day of the experiment. This pattern of FGA excretion is also shown in the photograph of the paper strips (Fig. 1) on which the com-

TABLE IV. Effect of Dietary Fat Level upon Excretion of FGA by Vit. B_{12} Deficient Chicks.*

Day	Dietary fat, %	Body wt, g	Histidine intake, mmole/day	μmole FGA Per day	μmole FGA Per mmole histidine
Exp. 1 (ad libitum)					
1	4	180	2.6	65	25
2	4	190	2.5	87	35
3	24	195	1.7	103	61
4	24	200	1.9	108	57
Exp. 2 (restricted food intake†)					
1	4	171	1.4	157	112
2	4	173	1.4	144	103
3	24	178	1.4	167	119
4	24	183	1.4	158	113

* 5 chicks in Exp. 1, 8 in Exp. 2; all 21 days old on day 1 of each experiment. All chicks received the low fat diet prior to 21 days of age.

† Amount of diet offered each chick each day was 75% of the avg amount consumed by the group on the day prior to day 1.

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ponents in extracts of excreta were separated electrophoretically. These were from one chick during successive days of the above experiment. No formimino-containing compound other than FGA has been detected during this work by either the chromatographic or electrophoretic procedures described above.

The chicks ate more of the diet on the day when the methionine supplement was given (Table II). This increase may have influenced the excretion of FGA on the day following supplementation. Food intake on the day of methionine supplementation was therefore limited to the same amount of diet as that eaten on the day prior to the methionine supplement (Table III). In this experiment methionine did not lower FGA to as low a level as that seen in Table II. A possible explanation may lie in the fact that the chicks ate all of their diet before the end of the 24 hr period and part of the "post-methionine" high rate of FGA excretion may have been included in this day's excretion. However, the rise in FGA excretion on the day following supplementation was similar to that in Table II.

Several compounds biochemically related to methionine were tested for their effect upon FGA excretion (Table III). Each was incorporated into the diet for one day at a level equimolar to the 1% supplement of methionine. The first 2 days of each of these experiments serve as the basis of comparison for assessing the effect of each of the supplements. None of these compounds (DL-homocysteine thiolactone HCl, DL-homocystine, L-cystine, choline chloride, or betaine) reduced FGA excretion. In fact FGA excretion was slightly higher on the day that betaine was given. When either homocystine or choline chloride was given, there was a moderate increase in mean FGA excretion on the day following the supplement; however, these effects were small when compared with those of methionine.

Chicks raised to 3 weeks of age on the Vit. B_{12}-free diet that contained only 4% of fat excreted large amounts of FGA (Table IV). When the fat content of this diet was increased to 24% there was a moderate increase in FGA excretion. There was a decrease in the weight of food ingested when the fat content of the diet was increased, probably due chiefly to the higher caloric density of the high fat diet. A second experiment was conducted in which food intake was restricted during the initial 2-day FGA measurement period. It was thus possible to keep food intake constant during the time that both the low and the high fat diets were fed. In this experiment there was no change in FGA excretion when the dietary fat intake was increased.

Discussion. The dramatic effect of a methionine supplement in decreasing the high rate of FGA excretion by Vit. B_{12} deficient chicks confirms the findings that Silverman and Pitney obtained with the rat(1). The mechanism of this effect has been discussed (1,7); however, it is still largely a matter for conjecture. In these experiments only the intact methionine molecule was effective. The very rapid effect of the methionine supplement as compared to that of Vit. B_{12} suggests that methionine may be directly involved in the metabolism of the formimino group arising from dietary histidine. The marked increase in FGA excretion following removal of the methionine supplement apparently was not related to the increased food intake that occurred when additional methionine was incorporated in the diet (Table III). A relatively small proportion of the dietary histidine was excreted as FGA by the deficient chicks. It is therefore possible that the Vit. B_{12} deficient chicks had become adapted to use efficiently the small amount of available methionine for metabolism of the formimino group and that this efficiency was temporarily lost when the 24 hr high methionine supplementation period ended. This may account for the observed excretion pattern (Table II, Fig. 1).

FGA excretion of individual Vit. B_{12} deficient chicks has been found to be fairly constant from day to day; however, somewhat larger differences in control values have been found between different experiments (days 1 and 2, Table III). These experiments were carried out over a period of several months and variability in the chicks may have been a factor. The 2 experiments reported in Table IV were conducted almost

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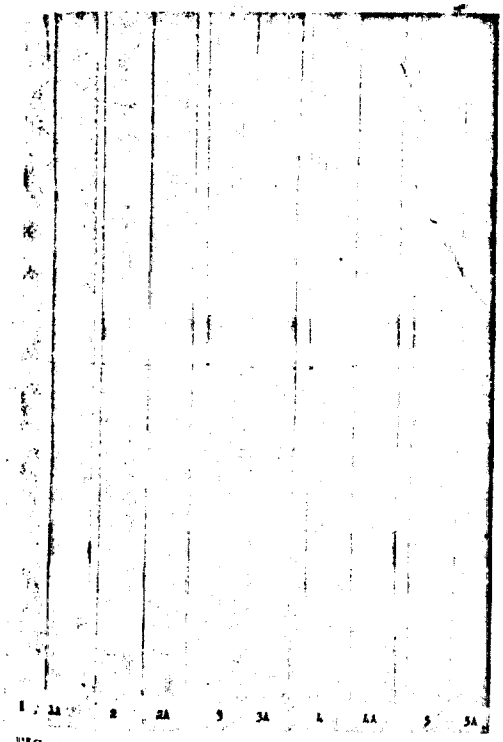

FIG. 1. Separation of amino acids by electrophoresis. The strip of each pair marked "A" was exposed to ammonia for 1 hr prior to staining with ninhydrin. The origin was marked with pencil on each side of each pair at the center of each strip. During electrophoresis the position of the lower ends of the strips as seen in the photograph were at the anode side. The patterns resulted from application of the following solutions: (1) standard FGA, 1 μmole/ml, (2) extract from vit. B_{12} deficient chick, 0.8 μmole FGA/ml (by colorimetric analysis), (3) same chick, day of 1% methionine supplement, 0.08 μmole FGA/ml, (4) same chick, first day after methionine supplement removed, 0.65 μmoles FGA/ml, (5) same chick, second day after methionine supplement removed, 0.9 μmole FGA/ml. Each day's excreta were made to a total volume of 50 ml.

successively. These data offer the suggestion that restriction of food intake may increase excretion of FGA. This possibility is being investigated further.

It is difficult to explain the differences in FGA excretion pattern between the 2 Vit. B_{12} deficient animal species and human subjects with pernicious anemia. Luhby *et al.* (8) and Spray and Witts(9) found that pernicious anemia patients excreted insignificant amounts of FGA after daily histidine loading tests of 15 and 2 g, respectively. In view of the results with animals, it is suggested that the methionine intake of the patients may have been sufficient to mask the effect of their Vit. B_{12} deficiencies upon FGA excretion. It is significant that a high level of dietary fat was not necessary to cause Vit. B_{12} deficient chicks to excrete large amounts of FGA. This is contrary to the earlier observations on growth of the chick fed this Vit. B_{12} deficient diet(3). The level of dietary fat is probably not involved in the differences between man and the chick.

Summary. Young chicks fed a diet deficient in Vit. B_{12} for 3-5 weeks excreted large amounts of formiminoglutamic acid (FGA) when compared to control chicks that received Vit. B_{12} in the diet. The high excretion rate of FGA by the Vit. B_{12} deficient chicks was reduced to very low levels by incorporation of 1% DL-methionine into the diet for 24 hr. This effect was not observed with supplements of DL-homocysteine thiolactone HCl, DL-homocystine, L-cystine, choline chloride, or betaine. Although a high level of dietary fat had previously been found necessary to cause poor growth in the absence of Vit. B_{12}, FGA excretion was quite high with either 4 or 24% of fat in the diet.

The authors wish to thank Dr. Milton Silverman for many helpful discussions and for the forminoglutamic acid that was used as a standard, and Mrs. Esther M. Hurley, Mrs. Helen M. Hood, and Mr. Woodrow W. Duvall for excellent assistance with these experiments.

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The basal diet was fed twice daily. It contained less than 1 μg. of vitamin B_{12} per pound, but not less than N.R.C. (9) recommended allowances of other nutrients. Animals 7 and 8 received no vitamin B_{12} supplement for the first 18 months and variable amounts until 2 months before conception. A supplement of 400 μg. of vitamin B_{12} daily was fed to animals 7 and 8 from 34 months of age and to animals 9-11 from 8 months of age. Animals 7-11 are designated hereafter as "treated" animals and animals 1-6 as "control" animals.

Animals were kept individually in pens on raised wooden floors covered with shavings. They were exercised twice weekly for 30 minutes on a mechanical exerciser that forced the pigs to walk at the rate of 3 m.p.h.

Blood samples were taken usually at 2-week intervals from the major blood-vessels in the region of the thoracic inlet. The 10 weeks before conception was considered the pre-gestation collection period. Reported values (Table 1) represent the mean serum vitamin B_{12} levels for the specified periods.

Piglets

Stillbirths, live births and neonatal deaths up to 3 days of age were recorded. Some of the piglets were removed from their dams on the third morning after birth. They were assigned to oral vitamin B_{12} supplementation tests as follows:

Piglet test 1

	Treated sows No.			
	7		8	
Oral vitamin B_{12} (μg./piglet/day)	0	20	0	20
Number of piglets	3	3	3	3
Observation period (days)	26	26	43	43

Piglet test 2

	Control sows					Treated sows			
	3		4		5	9		10	
Litter No.	1	2	1	2	1	1	2	1	2
Vitamin B_{12}/kg. dry matter intake (μg.)	No. piglets at each dosage level								
0	2	2	2	2	1	2	2	2	2
20	1	1	1	1	1	1	1	1	1
40	1	1	1	1	1	1	1	1	1
80	1	1	1	1	1	1	1	1	1

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Canadian Journ. Animal Sci. 41(2) 212-219 (1961)

SOME OBSERVATIONS ON THE RELATIONSHIP BETWEEN VITAMIN B_{12} AND REPRODUCTION IN SWINE[1]

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ABSTRACT

Six female swine were maintained during one or more gestations on a vitamin B_{12}-deficient diet. Two that had been fed the deficient diet for more than 2 years prior to their first gestation aborted. Four that received the deficient diet for 7 months prior to their first gestation farrowed a total of 8 litters containing 68 live piglets; 27 died within 3 days. All piglets fed a synthetic milk diet, deficient in vitamin B_{12}, died within 14 days.

Five adult females fed the deficient diet supplemented with 400 μg. of vitamin B_{12} per animal per day farrowed a total of 8 litters containing 83 live piglets; none died within 3 days. Of 14 piglets fed a vitamin B_{12}-deficient synthetic milk diet, 2 died within 14 days.

The different dietary vitamin B_{12} intakes of the adults influenced the serum vitamin B_{12} levels of the adults and of their offspring.

Piglets fed vitamin B_{12} gained more, but feed conversion was not influenced by the vitamin B_{12} dietary intake of the piglets or by the intake of their dams during gestation.

It is concluded that vitamin B_{12} influences swine reproduction and neonatal survival.

INTRODUCTION

During the past decade a number of reports have dealt with the influence of vitamin B_{12} on the reproduction of various species of animals, including swine. Most, but not all of the evidence indicates that vitamin B_{12} does influence reproduction, and that the dietary intake of vitamin B_{12} might be important for swine maintained under practical husbandry conditions (4).

The work reported here was carried out over a period of several years to clarify various aspects of the relationship between vitamin B_{12}, reproduction and neonatal performance.

EXPERIMENTAL

Adults

Eleven female swine, approximately 7 months old, received a vitamin B_{12}-deficient, corn-soybean meal diet (7), with or without a 400 μg. supplement of vitamin B_{12} per animal per day, for different periods of time prior to their first mating and during subsequent gestation periods according to the following plan:

	Basal diet						Basal diet plus vitamin B_{12}				
Sow no.	1	2	3	4	5	6	7	8	9	10	11
Age at 1st mating (months)	36	36	15	15	21	15	36	36	15	15	15
No. gestations studied	1	1	2	3	2	1	1	1	3	2	1

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Piglets in test 1 were grouped together in metal cages according to treatments and dams. Those in test 2 were kept in individual cages for either 53 or 56 days. All test piglets were fed six times daily between 7 a.m. and 8 p.m. a vitamin B_{12}-deficient, synthetic milk diet similar to the medium fat diet of Cunningham and Brisson (2). Modifications of the diet included the use of vitamin-free casein, adjustment of the solids content from 10 to 15 per cent, and deletion of the vitamin B_{12} supplement. A solution of crystalline vitamin B_{12} was administered *per os* to the treated piglets in test 1, and it was added to the milk fed to the treated piglets in test 2. Feed efficiencies were calculated as kilograms synthetic milk dry matter consumed/kilogram body weight gained.

Of the piglets that remained with the control dams, one-half received intramuscular injections of vitamin B_{12} soon after birth.

Blood samples were obtained by the method described for sows or by cardiac puncture.

Vitamin B_{12} Assay

Vitamin B_{12} was assayed with *Euglena gracilis var. bacillaris* (5).

Data were analysed by common statistical methods.

RESULTS AND DISCUSSION

Blood serum vitamin B_{12} levels of control sows were distinctly lower than those of treated sows (Table 1). This was true both prior to and during the different gestations.

TABLE 1. — BLOOD SERUM VITAMIN B_{12} LEVELS OF SOWS

Sow No.	Gestation	Serum vitamin B_{12} ($\mu\mu$g./ml.)				
		Pre-gestation	Days of gestation			
			0–28	29–56	57–84	85–42
		Control sows				
1	1	151	117	89	113	155
2	1	123	143	97	114	137
3	1	64	11	56	10	98
	2	—	95	—	132	84
4	1	136	83	70	37	84
	2	—	90	—	159	129
5	1	77	115	—	127	134
	2	—	—	—	117	101
6	1	<20	—	—	—	<20
		Treated sows				
7	1	437	224	254	257	290
8	1	274	195	162	228	—
9	1	544	564	315	362	438
	2	—	592	438	374	405
10	1	470	531	383	383	551
	2	—	431	403	417	443
11	1	280	—	—	—	326

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TABLE 2. — REPRODUCTIVE PERFORMANCE OF SOWS

Sow No.	Gestation	Farrowing history			Remarks
		Total born	Still-births	Neonatal deaths	
		Control sows			
3	1	12	0	1	
	2	12	2	3	
4	1	11	0	0	
	2	11	1	4	
	3	9	1	8	
5	1	11	2	3	
	2	9	3	6	
6	1	6	4	2	2 were deformed; 1 was born a day later.
Total		81	13	27	
		Treated sows			
7	1	13	7	0	Deaths were caused by anoxia resulting from prolonged delivery.
8	1	13	0	0	
9	1	15	2	0	
	2	15	1	0	
	3	10	2	0	The stillbirths were born a day later.
10	1	14	3	0	
	2	10	0	0	
11	1	11	3	0	
Total		101	18	0	

Sows 1 and 2, fed the deficient diet for the longest period prior to mating, aborted near term. Post-mortem examinations of their genital tracts revealed no evidence of infection.

The maternal dietary vitamin B_{12} status markedly influenced the survival of piglets born alive (Table 2). Clinical observations of successive litters born by basal-fed sows revealed progressively weaker newborn piglets. This is indicated only partially in Table 2; the effect was observed in offspring of sows maintained under practical conditions (6). Similar observations have been reported for rats (8) and mice (1). Piglets left with control dams survived only if they were given intramuscular injections of vitamin B_{12}.

Table 3 extends and corroborates the preceding observations on the effect of the different maternal and neonatal regimens on neonatal survival.

The original weight gain data revealed no apparent differences in the weight gains between piglets treated with 20, 40, or 80 μg. of vitamin B_{12} per kilogram of dry matter intake. Therefore the results for these treatments were grouped together. Variance analysis of mean litter weights of

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TABLE 3. — EFFECT OF MATERNAL AND PIGLET VITAMIN B_{12} DIETARY INTAKES ON PIGLET SURVIVAL AND 8-WEEK WEIGHT GAINS (PIGLET TEST 2)

Vitamin B_{12} in piglet diet (μg./kg. D.M.)	Control sows			Treated sows		
	Piglet weight gain (kg.)		Survival ratio[1]	Piglet weight gain (kg.)		Survival ratio[1]
	Mean	S. E.		Mean	S. E.	
0	—	—	0/9	15.7	1.1	7/8
20	19.5	0.5	3/5	20.6	1.6	4/4
40	17.7	3.5	3/5	21.7	1.4	3/4
80	19.5	1.4	3/5	19.4	1.3	3/4

[1]Number of survivors/number initially allocated

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treated piglets, either unweighted or weighted according to the number of piglets studied per litter, did not reveal any difference in piglet growth due to the maternal dietary vitamin B_{12} intake during gestation. Apparently, the deficiency acquired *in utero* was reversible in these treated piglets as far as weight gains were an indication, but this requires additional observations. It agrees with observations in mice (1) and rats (3, 8).

Comparison between final body weights of vitamin B_{12}-treated and untreated piglets born of treated sows were made on a within-litter basis, after adjustment for initial body weights. The mean difference for each litter was weighted inversely according to its variance, and the mean of these weighted differences was significant (P almost 0.001). Weight differences between treated and untreated piglets occurred in different litters at widely different times after allocation (2 to 8 weeks). The wide between-litter time differences may be part of the explanation for the widely different estimates of the vitamin B_{12} requirement of baby pigs reported in the literature.

In all groups there was a direct relationship between body weight gained and the amount of milk consumed. Average feed conversion efficiency for the different groups ranged between 7.2 and 7.7 kilograms dry matter consumed per kilogram body weight gained. Differences between groups were not apparent ($P > 0.05$).

The vitamin B_{12} dietary intake of dams influenced the blood serum vitamin B_{12} levels of piglets. Serum levels of three 3-day-old piglets born by control sows 3 and 4 ranged from 40 to 44 $\mu\mu$g./ml.; levels of four similar-aged piglets born by treated sows 9 and 10 ranged from 156 to 366 $\mu\mu$g./ml. Table 4 shows the effect of the neonatal dietary treatment of piglets born by treated sows 9 and 10 on serum levels at various intervals during the feeding trials. It also includes information from piglets born by treated sows 7 and 8. At each time interval the serum levels of untreated piglets were lower than those of treated littermates. Despite the different

TABLE 4. — EFFECT OF VITAMIN B_{12} SUPPLEMENTATION OF SYNTHETIC MILK DIET ON PIGLET BLOOD SERUM VITAMIN B_{12}($\mu\mu$g./ml.)[1]

Sow No.	Piglet age (weeks)	Piglet vitamin B_{12} supplement[2]			
		0	20	40	80
7	4	20-57 (3)[3]	239-724 (2)		
8	4	156-240 (2)	460-542 (2)		
	6	14-130 (2)	381-524 (2)		
9	8	162 (1)	265 (1)	231 (1)	258 (1)
10	8	134 (1)	260 (1)	258 (1)	191 (1)

[1]Data from piglet tests 1 and 2
[2]As μg./day for progeny of sows 7 and 8 and μg./kg. dry matter intake for progeny of sows 9 and 10
[3]Number of piglets in parentheses

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TABLE 5. — BODY WEIGHT GAINS (KG.) OF PIGLETS BORN OF TREATED SOWS 7 AND 8 (PIGLET TEST 2)

Sow	Vitamin B_{12} dose (μg./day)	
	0	20
7	3.0	3.0
	3.2	3.7
	5.6	—
	Mean 3.9	3.4
8	8.7	18.0
	6.8	16.3
	Mean 7.8	17.2

4-week serum levels of treated and untreatd piglets born by sow 7, differences in weight gains were not apparent at that time (Table 5). This agrees with our belief that serum levels are a more sensitive criterion of vitamin B_{12} status in the growing animal than weight gains. Weight gain differences between treated and untreated offspring born by sow 8 occurred as early as 16 days after birth. The 16-day body weights of piglets born by treated sow 8 were compared with similar data from the litters born by treated sows 9 and 10. When the mean litter differences in the 16-day body weights were adjusted for the initial weights, the mean difference between the treated and untreated piglets born by sow 8 was significant ($P < 0.05$). Blood samples were taken from 4-week-old piglets born by sow 8. Differences in serum vitamin B_{12} levels between treated and untreated piglets were apparent and the levels of the same untreated piglets declined further during the following 2 weeks.

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An Isotopic Measurement of Vitamin B_{12} in Cerebrospinal Fluid

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ABSTRACT

Frenkel, Eugene P., McCall, Mary Sue, and White, Jerry D.: An isotopic measurement of vitamin B_{12} in cerebrospinal fluid. Amer. J. Clin. Path. 55: 58–64, 1971. The competitive inhibition radioisotopic assay of serum vitamin B_{12} has been adapted for the measurement of cerebrospinal fluid B_{12}. The relatively low CSF B_{12} levels (1/20 to 1/30 that of serum) necessitated increased sensitivity. This was achieved by using a smaller amount of the labeled B_{12} for the competitive inhibition, prolonging the incubation time for interaction between B_{12} and the binding protein, and eliminating the potential for nonspecific binding by maintaining sterility. Proportional dilution studies demonstrated that normal CSF did not contain interfering substances. The range of normal was 8 to 36 pg. per ml. (mean 17.8 pg. per ml.). All of the patients deficient in vitamin B_{12} studied to date have had reduced CSF B_{12} values. There was no correlation between CSF B_{12} and CSF protein or serum B_{12}.

INTEREST in the relationship between vitamin B_{12} deficiency and neurologic lesions led to extensive laboratory studies of a variety of neural lesions following the advent of the microbiologic assay for B_{12}. The study of serum levels of B_{12} resulted in the identification of some deficient patients who had neural lesions in the absence of hematologic abnormalities. In light of this finding, several investigators[1, 2, 5–7, 9–14] have studied cerebrospinal fluid levels of B_{12}. Conflicting reports have beclouded the range of normal for CSF B_{12}, variously expressed as 0.01 to 0.1 pg. per ml. by Bauer and Heinrich[2] to 0 to 30 pg. per ml. by Sobotka and associates.[11] Similarly, patients with multiple sclerosis have been described as having large increases in CSF B_{12}[11, 12] or very low levels,[2] or normal values.[1, 10, 14] One basis for this conflict has related[1] to the different microorganisms (*Ochromonas malhamensis*,[11, 12] *Lactobacillus leishmanii*,[1] *Euglena gracilis*[1, 2, 10, 14]) utilized for this growth-dependent microbiologic assay.

The isotopic assay of B_{12} in serum has proven to be accurate and reproducible and has served as a valuable aid in the diagnosis of B_{12} deficiency.[3, 4, 8] A direct application of this competitive inhibition type of radioisotopic assay was hampered by the relatively low B_{12} values found in the CSF, which necessitated a 20-fold increase in sensitivity for accurate determination.

The present report describes a technic whereby concentrations as low as 1 pg. per ml. can be measured accurately. In addition, it delineates some of the relationships

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of CSF B_{12} to spinal fluid protein content and the serum B_{12} level. Data describing B_{12}-deficient states are also reported.

Methods

Reagents

1. Acetate buffer, pH 4.6, 0.625 M, diluted in I.V. water, and sterilized with millipore filtration.

2. Sodium cyanide, 25 μg. per ml., handled as above.

3. Acetate-CN-H_2O mixture: 1 volume acetate buffer, 2 volumes of NaCN, and 2 volumes sterile water. This was not combined until the day of use, and during mixing sterility was maintained.

4. Phosphate buffer, pH 8.0, 0.01 M sodium phosphate, prepared weekly, with working dilutions made daily with fresh distilled water.

5. Vitamin B_{12}, 1 ng. per ml. This standard reagent was diluted in the acetate-CN-H_2O mixture as described previously[3, 4, 8] and frozen in small samples suitable for both serum and spinal fluid assays. Prior to use in the CSF assay, a further dilution of 1:20 (50 pg. per ml.) was necessary.

6. $^{57}CoB_{12}$, specific activity 100 to 250 microcuries per mg. (Amersham/Searle Corp., Des Plaines, Ill. 60018, or N. V. Philips Duphar Co., Cyclotron and Isotopes Laboratories, Petten, Holland). Each new lot was assayed and checked for purity as previously described.[3, 4, 8] All dilutions were made in the acetate-CN-H_2O mixture and sterilized with millipore filtration before assay. Final dilution was 50 pg. per ml.

7. Standard serum. As with the serum assay[3, 4, 8] pooled human serum (Tissue Culture reagent, North American Biologicals, Inc., North Miami, Fla.) was used as a source of B_{12}-binding protein. The optimum quantity of this serum to be used in the subsequent spinal fluid assay was selected as follows: 5 pg. of $^{57}CoB_{12}$ (50 pg. per ml.) were diluted in 4 ml. of the acetate-CN-H_2O mixture in a Pyrex 25 by 125 mm. screw-capped test tube. (Unlike the routine standard curve procedure outlined below, boiling was not necessary for these initial determinations.) Unlabeled B_{12} (50 pg. per ml.) was added to enough tubes so that three or four standard curves could be run, all with different amounts of standard serum. In our experience the optimum amount usually has been 100 μl. of approximately a 1:10 dilution of the serum.

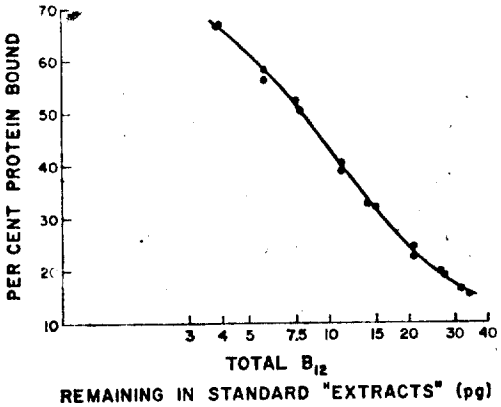

FIG. 1. A typical standard curve. After adding 5 pg. of $^{57}CoB_{12}$ to 0 to 45 pg. of unlabeled B_{12}, the standards were "extracted" or processed just as the CSF samples were (see text). After correcting for recovery, the individual pg. values shown on this two-cycle semilogarithmic scale were plotted.

After incubation overnight at room temperature, 10 ml. of phosphate buffer were added and the protein-bound B_{12} was adsorbed with 0.5 Gm. of cellulose in a manner identical to that used in the serum assay.[3, 4, 8]

An amount of standard serum which effects a slope most nearly approximating the curve shown in Figure 1 was selected. The standard serum was diluted in sterile water with sterile glassware to a volume which would yield a final optimum amount of serum in 100 μl. It was then frozen in measured quantities which were thawed daily.

Assay Procedure

As outlined in Table 1, 5 pg. of $^{57}CoB_{12}$ were added to 1 to 2 ml. of spinal fluid and B_{12} was extracted by boiling with acetate buffer and NaCN. As can be seen in this

Table 1. Assay Procedure for Determination of Cerebrospinal Fluid Vitamin B_{12} *

Standards	Unknowns
EXTRACTION	
2 ml. H_2O	2 ml. CSF (or 1 ml. CSF + 1 ml. H_2O)
Add 5.0 pg. (100 μl.) $^{57}CoB_{12}$	Same
Add 0 to 900 μl. standard B_{12} (0, 2.5, 5, 10, 15, 25, 35, and 45 pg.; volumes need not be constant). Note that unlabeled B_{12} is not added to the first tube, which already contains 5 pg. of labeled B_{12}	Equilibrate 30 min. at room temperature
Add 1 ml. acetate buffer and 2 ml. NaCN	Same
Boil 30 min.; cool to room temperature	Same
(Standards need not be centrifuged)	Centrifuge
Transfer 4 ml. supernatant with sterile pipette into sterile test tube	Same
ASSAY	
Add standard serum with sterile pipette	Same
Incubate 18 to 24 hr. at room temperature	Same
Any time during incubation, count and calculate % recovery (may range from 65 to 80%)	Same (usually between 65 and 75%)
Calculate *total* pg. amounts in each 4 ml.	Not applicable
Following incubation, add 10 ml. PO_4 buffer	Same
Add 0.5 Gm. cellulose and shake 15 min.	Same
Centrifuge and wash with PO_4 buffer 3 times	Same
Count packed cellulose	Same
Calculate % bound	Same
Plot standard curve (% bound *vs. total in each 4 ml.*) (Fig. 1)	Read values from curve; calculate pg./ml. CSF: $\text{pg. of } B_{12} = \dfrac{\left(\dfrac{\text{Amount of } B_{12} \text{ from curve}}{\text{recovery in percentage}}\right) - 5 \text{ pg.}^*}{\text{Volume of CSF assayed}}$

*The quantity of added $^{57}CoB_{12}$.

table, the standards were handled in an identical manner except that the 30-min. equilibration was not necessary.

Following extraction, 4 ml. of the extract were assayed and read from a standard curve which was always run with each series of samples. Since the percentage recoveries of the original standard mixtures varied, it should be noted that the known picogram values shown on the horizontal axis were the calculated values (Fig. 1).

Nonspecific Binding

The efficiency of endogenous CSF protein removal was evaluated by utilizing the routine assay with an extra tube in which the standard serum was omitted. Identifiable binding in this circumstance related either to the presence of endogenous B_{12}-binding protein or to the nonspecific binding of free B_{12} to cellulose. The latter effect was evaluated by use of a second control tube containing only the acetate-CN-H_2O mixture and 5 pg. of $^{57}CoB_{12}$. Binding identifiable by this method could be due only to the nonspecific effect of the cellulose.

Results

Nonspecific Binding

A successful competitive inhibition type of assay necessitated separation of B_{12} from endogenous CSF proteins and the complete

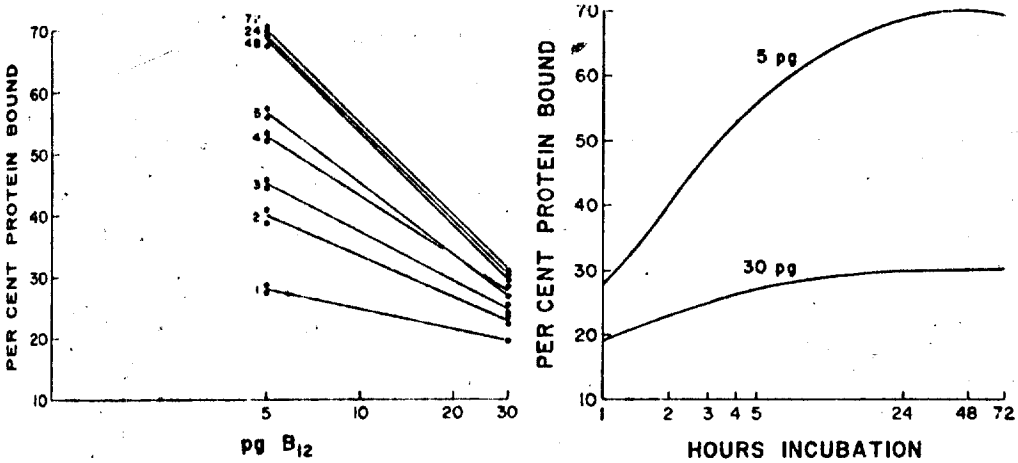

FIG. 2. Effect of incubation time. The figure on the left represents the effect of time on the slope of the curve. Five and 30 pg. of B_{12} were incubated for various periods from one to 72 hr. Prolonged incubation produced a curve with a good slope. The figure on the right depicts this data plotted in terms of time. The binding of the smaller quantity of B_{12} (5 pg.) appears more time dependent than that of the larger quantities (30 pg.). Incubation for more than 24 hr. did not significantly improve the slope of the curve.

removal of these proteins so that nonspecific binding, with resultant falsely low values, would not occur. In 32 assays the efficiency of endogenous protein removal in the extracts was evaluated and was consistently less than 3%. By the use of the control, containing only the acetate-CN-H_2O mixture and $^{57}CoB_{12}$, most of this binding was shown to be due to nonspecific binding to the cellulose. Since this cellulose effect presumably was identical in standards and in unknowns, no correction was required. Thus, since the true nonspecific binding to protein was generally less than 1%, no corrections were made for this minute difference.

Finally, it was noted that when nonsterile techniques were employed with our prolonged incubation times, the nonspecific cellulose binding was considerably increased.

Effect of Incubation Time upon Sensitivity

The CSF B_{12} level was approximately 1/20 to 1/30 that of serum. At these low levels, the slope of the standard curve was unsatisfactory since the overall decrease in binding was only approximately 10%.

In an attempt to achieve increased sensitivity by permitting maximum reactivity between the B_{12} and the B_{12}-binding proteins, various incubation times and B_{12} concentrations were studied. Five and 30 pg. of B_{12} were incubated with standard serum for 1, 2, 3, 4, 5, 24, 48, and 72 hours. As is evident in Figure 2, the slope of the

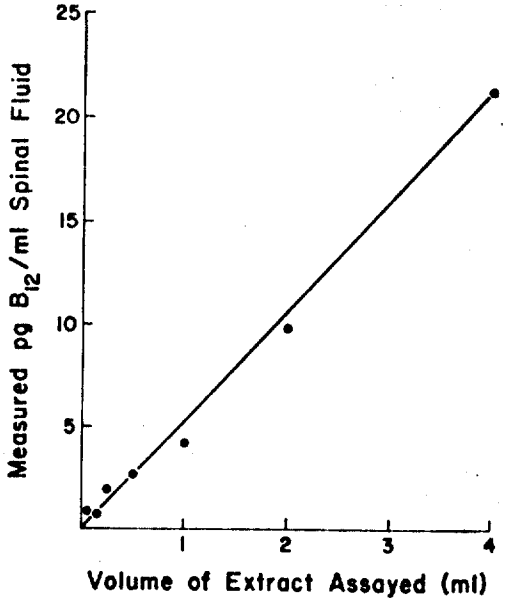

FIG. 3. Proportional dilution study. The extract was diluted in water. The proportionality delineated suggested the absence of interfering substances.

Table 2. Replicate Tests of a Single Spinal Fluid Sample

Assay Date	pg./ml. Assayed Duplicates	Average
11/25/68	4.7	4.8
	4.9	
12/1/68	5.1	5.0
	4.8	
1/9/69	5.4	5.4
	5.3	
1/16/69	6.6	6.7
	6.8	
1/27/69	6.3	6.0
	5.6	
3/24/69	5.8	5.6
	5.4	
9/29/69	6.0	5.5
	5.0	
11/3/69	4.3	4.3
	4.3	
MEAN		5.4
SD		0.695

curve increased as incubation time was prolonged, and the binding of the smaller quantity (5 pg.) of B_{12} appeared more time dependent than the binding of the larger (30 pg.) quantity. At 24 hours the binding appeared to reach a reasonable plateau; therefore, this time was selected as a valid and expedient incubation period. All subsequent data were obtained under these conditions.

Specificity

Specificity of the assay was evaluated by proportional dilution studies performed by simple dilution of the extract in water. The data of one representative study, as noted in Figure 3, suggested that the measured concentration decreased linearly with sample dilution. These studies demonstrated that the calculated concentrations of undiluted spinal fluid were the same at all dilutions assayed. This suggested the absence of substances in spinal fluid which interfere with the kinetics of binding. These dilution relationships were not obtained unless the standards were boiled and processed exactly as the unknowns and unless sterile solutions were utilized.

Reproducibility

Daily reproducibility is recorded in Table 2. On many occasions over a 12-month period, frozen samples of appropriately diluted spinal fluid was run as controls. As demonstrated, the average value for this pool was 5.4 pg. per ml. (SD = 0.695).

Patient Studies

Cerebrospinal fluid B_{12} levels of 62 patients are shown in Figure 4. These "normals" were obtained from patients with no neurologic involvement and normal hematologic and cerebrospinal fluid laboratory values. Most of these patients were being evaluated for low back strain or elective surgery with spinal anesthesia. The mean CSF B_{12} of the 62 patients was 17.80 pg. per ml. (SD = 7.79, SE = 0.98).

Thirteen patients with pernicious anemia (P.A.) were studied. Cases were documented by classical clinical and laboratory data and hematologic responses to physio-

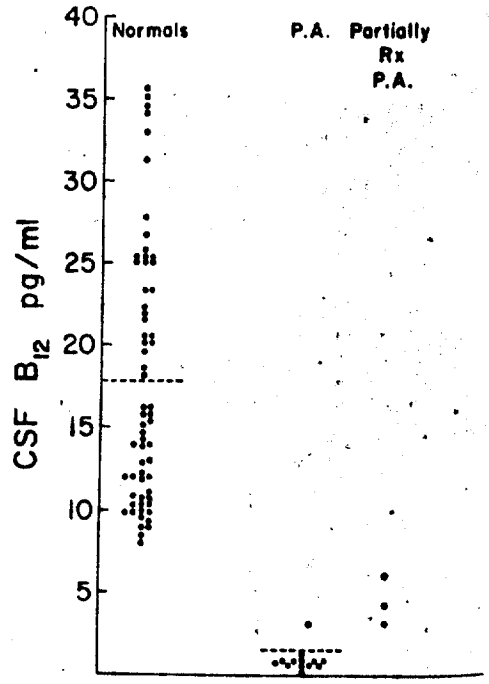

FIG. 4. Range of values. The ranges of values for normals, for patients with pernicious anemia, and for patients with "partially treated" pernicious anemia are shown.

logic amounts of parenteral cyanocobalamin. As demonstrated in Figure 4, the mean CSF B_{12} for these patients was 1.5 pg. per ml. (SD = 1.7, SE = 0.4). Most of the values were below 3 pg. per ml. Three patients with known pernicious anemia, partially treated with B_{12}, are also represented in Figure 4. These patients are described as "partially treated" since they were known to have P.A. and had received B_{12} at various times in the distant past but had been lost to follow-up and appropriate serial therapy. None of these patients had megaloblastosis, although hypersegmentation of the polymorphonuclear (PMN) leukocytes and a slight shift in the mean lobe (PMN) average was present in one patient.

Relationship of CSF B_{12} to Serum B_{12}

The relationship of the serum B_{12} values to CSF B_{12} in normal and P.A. patients is depicted in Figure 5. As can be seen, a direct relationship between serum and CSF B_{12} values was not evident in the normal patients. The three "partially treated" P.A. patients were of particular interest.

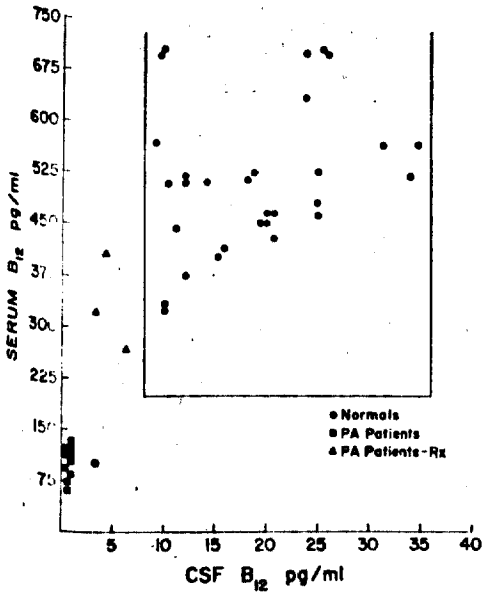

FIG. 5. Relationship between serum and CSF B_{12}. The CSF and serum B_{12} values for normal patients are plotted. The enclosed box indicates our normal ranges for serum and CSF B_{12}.

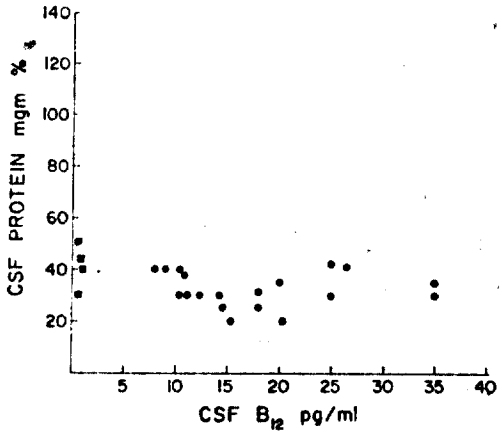

FIG. 6. Relationship of CSF B_{12} to CSF protein. Circles represent the normal patients; patients with pernicious anemia are designated by squares.

Their CSF B_{12} values were clearly below our range of normal, although their serum B_{12} values were normal (260, 325, and 420 pg. per ml.).

Relationship between CSF B_{12} and CSF Protein

No relationship between CSF B_{12} values and cerebrospinal fluid protein values was found in either P.A. patients or normal patients (Fig. 6).

Discussion

The radioisotopic assay for serum vitamin B_{12} has proved to be accurate, rapid, and relatively simple.[3, 4, 8] The application of this method to cerebrospinal fluid appeared highly desirable because of the variability of data based upon the microbiologic assay which had been recorded previously.[1, 2, 5, 7, 9–14] To make such an assay useful for cerebrospinal fluid, adaptation to accommodate the very low CSF values in normal persons was necessary. The CSF B_{12} normally appears to be 1/20 to 1/30 of the serum value. To identify these low levels, an increase in sensitivity was achieved by permitting prolonged equilibration between the B_{12} and the added binding protein, similar to the technic employed in most radioimmunoassay methods. For this prolonged incubation pe-

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riod to be effective, sterility was necessary, since bacterial contamination provided nonspecific protein for binding to the B_{12}.

The assay method has proven to be reproducible. Proportional dilution studies (Fig. 3) suggest that at least normal spinal fluid does not contain materials which interfere with the kinetics of binding.

As in serum, the range of normal for CSF B_{12} appears broad. It is noteworthy that there is no direct relation between CSF B_{12} and CSF protein. Previous studies by Kidd and co-workers[7] produced similar results using the microbiologic assay; these authors suggested that the B_{12} in CSF is not actually protein-bound. Worm-Petersen,[14] however, had described a relationship between serum B_{12} levels and the CSF values. Such a relationship has not been found by others in the microbiologic assay,[7] and it is not evident by the sensitive radioisotopic method. Relative to this, the three partially treated P.A. patients (Fig. 5) are of interest. Despite the fact that two of the three had serum values equal to or greater than normal, their CSF values were very low. This suggests that a simple threshold phenomenon for CSF B_{12} does not exist.

There is very little information concerning the transport of vitamin B_{12} into the CSF. Because of the concentration difference between the two compartments, Bauer and Heinrich[2] studied the mechanics of reverse transport, that is, from CSF into the serum. At least this directional flow appeared to be slow. Kidd and co-workers[7] simply concluded that a "selective control" of passage of B_{12} across the blood–CSF barrier exists, and thus explained the relatively low B_{12} values in normal cerebrospinal fluid as well as the disparity between serum and CSF values. We cannot improve upon this suggestion.

To date, all of our patients who have pernicious anemia, documented by complete clinical and laboratory evaluation and corroborated by responses to physiologic amounts of vitamin B_{12}, have had very low CSF B_{12} values.

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COMPARISON OF ANTIGENIC AND CHEMICAL SIMILARITIES BETWEEN VITAMIN B_{12}-BINDERS OF HUMAN GASTRIC JUICE AND VITAMIN B_{12}-BINDERS EXTRACTED FROM THE MUCOSA OF CHIMPANZEE (*PAN TROGLODYTES*) STOMACH

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SUMMARY

Normal human gastric juice and an extract of chimpanzee fundus were studied by chromatography on Sephadex G-100 columns. Two B_{12}-binders were demonstrated in both human gastric juice and chimpanzee fundus extract, one which eluted with proteins in the mol. wt. range of 90000, the second which eluted with proteins having a mol. wt. of approx. 50000. The small B_{12}-binder in both human gastric juice and chimpanzee fundus extract reacted with the two antibodies found in serum of pernicious anemia patients that neutralize human intrinsic factor. When chimpanzee fundus extract was used as a source of intrinsic factor to determine the titer of the antibody in pernicious anemia serum which neutralizes the B_{12} combining site of intrinsic factor, identical titers were obtained as when human gastric juice was used as the source of intrinsic factor.

INTRODUCTION

The presence of an auto-antibody which reacts with intrinsic factor (IF) has been demonstrated in the serum of many patients with pernicious anemia, using both techniques *in vivo* and *in vitro*. JEFFERIES, HOSKINS AND SLEISENGER[1] using the bioassay of Schilling, demonstrated that an auto-antibody which destroyed the intrinsic factor activity of human gastric juice was present in about 30 % of the pernicious anemia sera they tested.

Several techniques *in vitro* have been used to assay for intrinsic factor auto-antibody and these methods have recently been reviewed by ROITT, DONIACH AND SHAPLAND[2]. The latter presented evidence in support of the concept that two anti-IF-auto-antibodies may exist; one antibody, combining site antibody (CSAB), which blocks the combination of vitamin B_{12} to intrinsic factor; the second antibody (CAB), which reacts with the vitamin B_{12}-intrinsic factor complex (IF-B_{12}). These auto-

Abbreviations: IF, intrinsic factor; CSAB, combining site antibody; CAB, complex antibody; IF-B_{12}, vitamin B_{12}-intrinsic factor complex.

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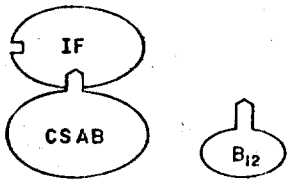
antibodies are diagrammatically represented in Figs. 1 and 2. IMRIE AND SCHILLING[3] have also presented similar evidence. Methods for the assay *in vitro* of the antibody (CAB) which reacts with the IF–B_{12} complex have been developed using starch-gel electrophoresis[2], $(NH_4)_2SO_4$ precipitation[4], and zirconyl-phosphate gel[5]. The antibody



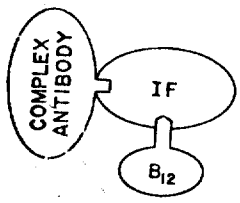


Fig. 1. A diagrammatic representation of CSAB.

Fig. 2. A diagrammatic representation of CAB.

(CSAB) which reacts with the B_{12}-combining site of intrinsic factor has been measured by three techniques *in vitro*. ABELS *et al.*[6], have utilized dialysis to remove free vitamin B_{12}, and thus assay pernicious anemia sera for CSAB. ARDEMAN AND CHANARIN[8], have used the 'charcoal method', which removes free vitamin B_{12} by adsorption with charcoal[7], for this auto-antibody. GOTTLIEB *et al.*[9] have also modified the charcoal assay of MILLER to assay for CSAB. HANSEN AND MILLER have modified a method which they developed to predict the IF activity of porcine intrinsic factor concentrates using zirconyl phosphate gel[10] to assay for CSAB (ref. 11).

It has been recognized for some years that intrinsic factor strongly binds vitamin B_{12} and methods have been available to differentiate between bound and free vitamin B_{12}. However, the assay of intrinsic factor activity of the human gastric juice by methods *in vitro* has been complicated by the presence of a non-specific vitamin B_{12}-binder (or -binders), in gastric juice from both normal subjects and patients with pernicious anemia[12]. These binders have properties very similar to intrinsic factor, and in some cases, the concentration of vitamin B_{12}-binders of gastric juice obtained from pernicious anemia patients or normal controls prior to histamine stimulation is high or higher than that of normal gastric juice obtained after histamine stimulation. CSAB has been used to differentiate between intrinsic factor and the 'non-specific' vitamin B_{12}-binders in human gastric juice[12]. GRASBECK AND SIMONS have studied the vitamin B_{12}-binders of human gastric juice and have found that intrinsic factor activity is associated with a binder which has a mol. wt. in the order of 50000–60000 (refs. 13–16). They have characterized a second vitamin B_{12}-binder which does not possess intrinsic factor activity and has a mol. wt. in the order of 90000–110000. They have designated this binder as 'R' and have demonstrated it to be present in serum, saliva and in gastric juice obtained from pernicious anemia patients.

In a previous communication, we have reported that CAB and CSAB react with intrinsic factor of human gastric juice but do not react with R-binder[7]. This has been recently confirmed by other investigators[18,19]. This report deals with a comparison of the vitamin B_{12}-binders of human gastric juice with the vitamin B_{12}-binders extracted from the gastric mucosa of the chimpanzee and the cross reaction of CAB and CSAB with chimpanzee vitamin B_{12}-binders.

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MATERIALS AND METHODS

Normal human gastric juice

Healthy young male adults were fasted for 12 h and the contents of their stomachs aspirated as completely as possible. Histalog (50 mg) was then administered subcutaneously and gastric juice obtained 15 min and 30 min later. The gastric juice was immediately adjusted to pH 7.4 with NH_4OH and the two specimens then pooled. The pooled gastric juice was centrifuged at 30000 × *g* (5°) for 1 h, the supernatant decanted from any mucous or particulate matter, immediately frozen in small aliquots and stored at −20°. The gastric juice was thawed in cold running water and in no experiment was it re-frozen.

Preparation of chimpanzee fundus gastric mucosa extract

The entire stomach was removed from a *Pan troglodytes* immediately after death. The stomach was washed with cold saline, the fundus mucosa scraped from the stomach wall, weighed and homogenized for 5 min in a Waring blendor with 5 parts (w/v) cold saline. The homogenate was then centrifuged at 8000 × *g* for 20 min at 5°. The supernatant was pooled and adjusted to pH 5.0 with 1.0 M acetic acid. The precipitate was removed by centrifugation at 8000 × *g* for 20 min at 5°, the supernatant pooled, immediately frozen in small aliquots and stored at −20°. The extract was thawed in cold running water and in no experiment was it re-frozen and employed subsequently.

Assay for CSAB (ref. 11), and CAB (ref. 5), and IF (ref. 12), have been described elsewhere.

Chromatographic separation of human gastric juice and chimpanzee mucosal extracts

Human gastric juice and mucosal extracts were chromatographed on a Sephadex G-100 column of packed dimensions of 45 cm × 4 cm. All separations were performed at 5°, using normal saline as the effluent. In all experiments 5.6 ml total volume was chromatographed with a flow rate of 0.6 ml/min and 5-ml fractions collected. Two ml of bovine serum which had been pre-mixed with 0.4 mg of unlabeled vitamin B_{12} was included in each separation as a molecular size marker. Control studies demonstrated that the bovine serum saturated with unlabeled vitamin B_{12} did not interfere with the distribution of labeled vitamin B_{12}.

RESULTS

The chromatographic separation of normal histamine-stimulated human gastric juice treated with excess [^{57}Co]vitamin B_{12} (obtained from E.R. Squibb and Sons, specific activity 1 μC/0.75 μg) is shown in Fig. 3. The two absorbance peaks represent from left to right; serum globulins of mol. wt. 100000–800000 and serum albumin of mol. wt. 65000. Factor R, a vitamin B_{12}-binder without IF activity, accounts for about 25 % of the [^{57}Co]vitamin B_{12} bound by the human gastric juice and elutes from the column in the region of compounds with 90000 mol. wt.; IF accounts for the remaining 75 % of the vitamin B_{12}-binding capacity of the gastric juice, and elutes from the column in the region of compounds with 50000 mol. wt., just after serum albumin. Unlabeled vitamin B_{12} and excess [^{57}Co]vitamin B_{12} did not elute from the column until 475 ml of effluent had passed.

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When the same gastric juice was treated with pernicious anemia serum, known to contain CSAB, before addition of [^{57}Co]vitamin B_{12}, bovine serum and unlabeled vitamin B_{12}, and the mixture chromatographed as outlined above, the separation seen in Fig. 4 was obtained. The IF–[^{57}Co]vitamin B_{12} peak disappeared, indicating that the CSAB in the serum completely neutralized the vitamin B_{12} binding site of IF. R–[^{57}Co]vitamin B_{12} remained unchanged, which indicated no reaction of this protein with CSAB. All [^{57}Co]vitamin B_{12} other than R–[^{57}Co]vitamin B_{12} eluted with unlabeled vitamin B_{12} but is not shown in this figure.

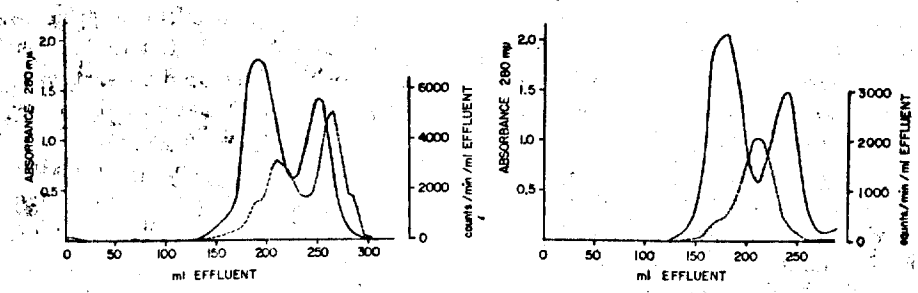

Fig. 3. Human gastric juice. Sample applied to Sephadex G-100 column: 3.0 ml human gastric juice, 1 μg [^{57}Co]vitamin B_{12} (0.2 ml), 0.4 mg unlabeled vitamin B_{12} (0.4 ml), 2.0 ml bovine serum. Human gastric juice, and [^{57}Co]vitamin B_{12} mixed before addition of unlabeled vitamin B_{12} and bovine serum. Solid line represents absorbance, broken line represents radioactivity. See text for details.

Fig. 4. Human gastric juice incubated with CSAB. Sample applied to Sephadex G-100 column: 3.0 ml human gastric juice, 2.0 ml CSAB containing pernicious anemia serum, 1 μg [^{57}Co]vitamin B_{12} (0.2 ml), 0.4 mg unlabeled vitamin B_{12} (0.4 ml). Human gastric juice and pernicious anemia serum mixed and incubated before [^{57}Co]vitamin B_{12} and unlabeled vitamin B_{12} were added. Solid line represents absorbance, broken line represents radioactivity. See text for details.

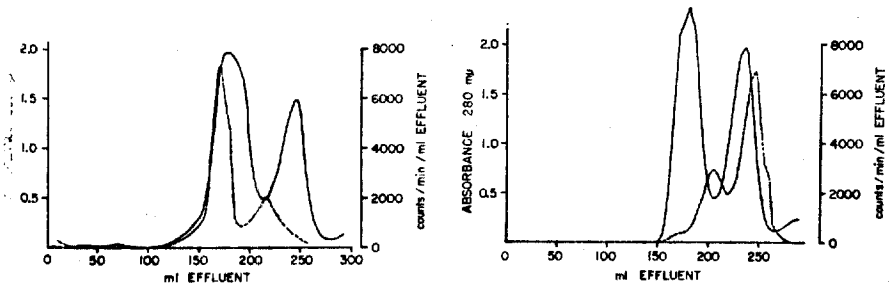

Fig. 5. Human gastric juice incubated with [^{57}Co]vitamin B_{12} then with CAB. Sample applied to Sephadex G-100 column: 3.0 ml human gastric juice, 1 μg [^{57}Co]vitamin B_{12} (0.2 ml), 2.0 ml CAB containing pernicious anemia serum, 0.4 mg unlabeled vitamin B_{12} (0.4 ml). Human gastric juice and [^{57}Co]vitamin B_{12} mixed before addition of pernicious anemia serum and unlabeled vitamin B_{12}. Solid line represents absorbance, broken line represents radioactivity. See text for details.

Fig. 6. Chimpanzee fundus extract. Sample applied to Sephadex G-100 column: 3.0 ml chimpanzee fundus extract, 1 μg [^{57}Co]vitamin B_{12} (0.2 ml), 0.4 mg unlabeled vitamin B_{12} (0.4 ml), 2.0 ml bovine serum. Chimpanzee fundus extract and [^{57}Co]vitamin B_{12} mixed before addition of unlabeled vitamin B_{12} and bovine serum. Solid line represents absorbance, broken line represents radioactivity. See text for details.

The vitamin B_{12}-binders of human gastric juice were saturated with [^{57}Co]-vitamin B_{12} and the mixture treated with the serum of a pernicious anemia patient which was known to contain CAB and fractionated as above. The results are shown in Fig. 5. R–[^{57}Co]vitamin B_{12} again remained, which indicates that factor R does not react with CAB. Again the IF–[^{57}Co]vitamin B_{12} peak completely disappeared; however, a new peak containing about 75 % of the bound [^{57}Co]vitamin B_{12} was found in the 'break-through' region and was presumed to represent the CAB–IF–[^{57}Co]-vitamin B_{12} complex which would be expected to have a mol. wt. greater than 100000.

The mucosal extract prepared from the fundus region of the chimpanzee stomach contained vitamin B_{12}-binding substances of molecular size which appeared to be identical to the human gastric juice vitamin B_{12}-binders, IF and R. Results from the Sephadex chromatography of this extract, which had been treated with excess [^{57}Co]vitamin B_{12} and bovine serum saturated with unlabeled vitamin B_{12}, are shown in Fig. 6. Two peaks which contained [^{57}Co]vitamin B_{12} were present. About 25 % of the bound [^{57}Co]vitamin B_{12} was associated with a protein which eluted from the column between the globulins and albumin. The remaining 75 % of the bound vitamin B_{12} was present in a peak which corresponded to a protein of mol. wt. of about 50000 and is similar to IF of human gastric juice.

When chimpanzee fundus extract was treated with pernicious anemia serum containing CSAB before addition of [^{57}Co]vitamin B_{12} and the resultant complex was treated as previously described, the results shown in Fig. 7 were obtained. The IF peak disappeared implying that human CSAB neutralized the vitamin B_{12}-binding site of chimpanzee IF. R–[^{57}Co]vitamin B_{12} remained unchanged which indicated that factor R did not cross react with the human auto-antibodies.

The chimpanzee fundus extract was then treated with [^{57}Co]vitamin B_{12} and subsequently with a pernicious anemia serum containing CAB. The results of the fractionation on Sephadex G-100 are shown in Fig. 8. R–[^{57}Co]vitamin B_{12} again

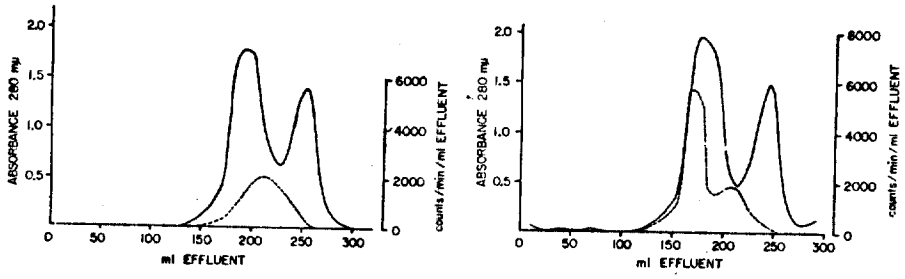

Fig. 7. Chimpanzee fundus extract incubated with CSAB. Sample applied to Sephadex G-100 column: 3.0 ml chimpanzee fundus extract, 2.0 ml CSAB containing pernicious anemia serum, 1 μg [^{57}Co]vitamin B_{12} (0.2 ml), 0.4 mg unlabeled vitamin B_{12} (0.4 ml). Chimpanzee fundus extract and pernicious anemia serum mixed and incubated before [^{57}Co]vitamin B_{12} and unlabeled vitamin B_{12} were added. Solid line represents absorbance, broken line represents radioactivity. See text for details.

Fig. 8. Chimpanzee fundus extract incubated with [^{57}Co]vitamin B_{12}, then with CAB. Sample applied to Sephadex G-100 column: 3.0 ml chimpanzee fundus extract, 1 μg [^{57}Co]vitamin B_{12} (0.2 ml), 2.0 ml CAB containing pernicious anemia serum, 0.4 mg unlabeled vitamin B_{12} (0.4 ml). Chimpanzee fundus extract and [^{57}Co]vitamin B_{12} mixed before addition of pernicious anemia serum and unlabeled vitamin B_{12}. Solid line represents absorbance, broken line represents radioactivity. See text for details.

remained unchanged; but the IF–[^{57}Co]vitamin B_{12} peak disappeared. A new peak appeared in the mol. wt. range of 100000 or greater which contained about 75 % of bound [^{57}Co]vitamin B_{12} and presumably, therefore, represents CAB–IF–[^{57}Co]-vitamin B_{12} complex. Chimpanzee IF cross reacted with the human auto-antibodies indicating that human and chimpanzee IF have common antigenic determinants.

TABLE I

COMPARISON OF REACTION OF ANTI-IF ANTIBODIES FOUND IN SERUM OF PERNICIOUS ANEMIA PATIENTS WITH IF ACTIVITY OF HUMAN GASTRIC JUICE AND IF ACTIVITY OF CHIMPANZEE FUNDUS EXTRACT

*Pernicious anemia patient**	*CSAB titer***	
	vs. human gastric juice	*vs. chimpanzee fundus extract*
E.B.	803 (790)***	788 (785)
F.J.	201 (198)	223 (230)
E.B.B.	1376 (1400)	1284 (1300)
I.C.	182 (178)	187 (190)
R.R.	459 (455)	450 (455)

* Diagnosis established by Schilling Assay, complete absence of IF in gastric juice and presence of anti-IF antibodies in serum.
** Antibody which reacts with vitamin B_{12} combining site of IF.
*** Results of duplicate experiment in parentheses.

Further evidence supporting the antigenic similarity of human and chimpanzee IF is shown in Table I. Pernicious anemia sera were assayed for CSAB using chimp fundus extract as a source of intrinsic factor, and the titers compared with those obtained when human gastric juice was used as the source of IF. Using the method of least squares, a 95.1 % correlation in the titers was obtained, indicating significant cross reaction. Neither IF from human gastric juice, nor IF of chimpanzee fundus extract reacted with normal human serum or serum of patients with macrocytic anemia of non-pernicious anemia orgin.

DISCUSSION

Chromatography of histamine stimulated human gastric juice saturated with [^{57}Co]vitamin B_{12} on Sephadex G-100 columns resulted in separation of two vitamin B_{12}-binders. Approx. 75 % of the bound [^{57}Co]vitamin B_{12} is associated with IF which has an apparent molecular size of 50000 mol. wt. proteins. A second binder R representing about 25 % of the original vitamin B_{12}-binding activity, elutes from G-100 between albumin and globulin and has an apparent molecular size of 90000 mol. wt. proteins. As discussed above, GRASBECK AND SIMONS[13-16] have isolated similar binders from human gastric juice. The smaller binder reacts with both of the anti-IF antibodies found in the serum of many patients with pernicious anemia, *e.g.* the antibody (CSAB) which neutralizes the vitamin B_{12} combining site of IF, and the antibody (CAB) which combines with IF–B_{12}. Neither antibody reacts with R.

The S_4 fraction obtained after differential centrifugation of chimpanzee fundus homogenates chromatographed on Sephadex G-100 demonstrates vitamin B_{12}-binders

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almost identical to those found in human gastric juice. About 75 % of bound vitamin B_{12} is associated with a protein of molecular size about 50000 mol. wt. proteins, a substance which reacts with both human IF auto-antibodies. The cross reaction of CSAB with human gastric juice IF and chimpanzee fundus extract IF appears to be complete.

KUBO, KAKEI AND GLASS[20] and ROTHENBERG[21] have studied normal human gastric juice by gel filtration and have come to the conclusion that the mol. wt. of IF is over 200000 since they found it to be excluded by Sephadex G-200 columns. However, in both of these cases, the gastric juice was processed by procedures which conceivably could have resulted in polymerization of IF–B_{12}, and in neither report was there indication that the columns were standardized with proteins of known molecular weight to assure that separation of 50000–200000-mol. wt. proteins could be obtained.

ACKNOWLEDGEMENTS

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Relation between Vitamin D and Vitamin B_{12}

In some experiments on chick growth with wholly vegetable diets[1] supplemented with pure vitamin B_{12}, a relationship became evident between the growth-stimulating effect of this vitamin and the content of calcium, iron and vitamin D of the diet. In an attempt to elucidate the relation between the vitamin D and vitamin B_{12}, the present experiment was started.

Day-old White Leghorn chicks from non-depleted hens were, during their first week, given a wholly vegetable diet (207 in Table 1), without added vitamin D. Then they were weighed and according to their weight grouped in six equal groups, each consisting of four sub-groups with nine chicks. They were fed the same diet with different vitamin D and vitamin B_{12} supplements. The experimental period lasted three weeks, and the results are given together with the experimental plan in Table 2.

Table 1. Percentage Composition of Experimental Diet 207

Barley	22·3
Oats	14·0
Wheat bran	11·0
Corn meal	15·1
Soybean oil meal	16·6
Sunflower seed meal	10·5
Alfalfa meal	4·0
B-vitamin conc.*	1·0
Bone meal	2·0
Ground limestone	1·6
Salt mixture†	1·0
	100·0

* Minimum 200 mgm. riboflavin, 200 mgm. niacin and 20,000 I.U. thiamin per kgm.

† 896 gm. iodized NaCl (5 mgm. KI per kgm.), 50 gm. $MnSO_4.4H_2O$, 50 gm. $FeSO_4.7H_2O$, 4 gm. $CoSO_4.7H_2O$.

Table 2. CHICK GROWTH AND FEED CONSUMED PER UNIT GAIN (UNTIL FOUR WEEKS OF AGE)

Group No.	Vitamin D supplement			Vitamin B_{12} (μgm. per kgm. diet)	Gain‡		Feed per kgm. gain (kgm.)
	Cod liver oil* %	Deltafor D_2—400† %	Approx. I.U. per kgm. diet		abs. (gm.)	rel. %	
1	0	0·05	200	0	128	100	3·06
2	0	0·05	200	30	134	105	3·01
3	0·4	0·1	640	0	110	86	3·45
4	0·4	0·1	640	30	141	110	3·03
5	0·4	0·3	1,490	0	106	83	3·29
6	0·4	0·3	1,490	30	144	112	3·04

* 1,000 I.U. vitamin A, 60 I.U. vitamin D per gm.
† 400 I.U. vitamin D_2 per gm.
‡ Gain between one and four weeks, average males–females.

From this table it is evident that the two higher vitamin D supplements had significant growth-depressing effects in the groups with no vitamin B_{12}. Supplementing the diet with vitamin B_{12}, however, eliminated these effects.

Details of this and other experiments in this series will be published elsewhere.

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May 7.

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VITAMIN B_{12} ABSORPTION: RELATIONSHIP BETWEEN ORAL ADMINISTRATION AND URINARY EXCRETION OF COBALT^{60}-LABELED CYANOCOBALAMIN FOLLOWING A PARENTERAL DOSE

STUDY OF DOSES OF 2 TO 250 μg IN 148 APPARENTLY HEALTHY MEN 20 TO 92 YEARS OLD

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ABSORPTION of orally administered radioactive cobalt-labeled vitamin B_{12} (cyanocobalamin or derivative) has been estimated in normal human subjects by difference in the amount of radioactivity administered and that recovered in the feces.[1-5] Absorption has also been correlated with hepatic accumulation[6] and plasma levels[7-10] following oral administration of labeled vitamin B_{12}, and with radioactivity appearing in the urine after oral administration of the labeled vitamin followed by a large parenteral dose of unlabeled material.[11] Correlations between urinary excretion of absorbed vitamin B_{12} and plasma levels[9] and between results from fecal and urinary excretion tests[12] have been demonstrated. The reliability of the urinary excretion technique for distinguishing between normal and markedly impaired absorption of vitamin B_{12}, for smaller oral doses (from 0.1 to 2 μg, usually about 0.5 μg), has been confirmed in many laboratories,[3, 4, 7, 12-16] although abnormally low excretions of radioactive vitamin B_{12} have been noted in terminal cardiorenal disease[7] and in severe renal disease.[17]

The present study was designed, primarily, to evaluate, in healthy subjects, the effect on urinary recovery following a parenteral dose, of varying the oral dose of vitamin B_{12} over a wide range, extending from physiologic or near-physiologic levels to several hundred times these levels. A second consideration has been exploration for possible age differences in absorption for such doses. The dose levels we have selected for study (2 to 250 μg) are higher than those at which an intrinsic factor deficiency, if existent, would seem most likely to be detected, since smaller overlap between results for normal subjects and pernicious anemia patients has been observed when the oral dose was less than 1 μg than when a larger dose was used.[4, 18, 18] Thus, for the higher doses of the present study at least, exploration for age differences constitutes study of agewise variation in efficiency of absorption via mechanisms relatively independent of the amount of intrinsic factor available. While furnishing information on a

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TABLE I. RENAL FUNCTION STUDIES FOR FOURTEEN ELDERLY SUBJECTS IN PRESENT STUDY (VALUES FROM LITERATURE FOR AGEWISE COMPARISON)

	ELDERLY SUBJECTS OF PRESENT STUDY									HEALTHY MEN STUDIED BY DAVIES AND SHOCK[19]*					
AGE WHEN RENAL FUNCTION STUDIED (YEARS)	NUMBER OF SUBJECTS	INTERVAL BETWEEN RENAL FUNCTION AND PRESENT STUDIES (YEARS)		INULIN CLEARANCE (ML./MIN./1.73 M.²)		DIODRAST OR PAH CLEARANCE (ML./MIN./1.73 M.²)		Tm DIODRAST OR PAH (MG. I/MIN./1.73 M.²)		INULIN CLEARANCE (ML./MIN./1.73 M.²)		DIODRAST CLEARANCE (ML./MIN./1.73 M.²)		Tm DIODRAST (MG. I/MIN./1.73 M.²)	
		MEAN	RANGE	MEAN	RANGE	MEAN	RANGE	MEAN	RANGE	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.
20-29	–									123	16	614	75	54.6	9.5
30-39	–									115	11	649	117	51.0	8.6
40-49	–									121	23	574	112	49.9	9.8
50-59	1	5	–	85.8	–	481†	–	74.7†	–						
		3	–	96.0	–	432	–	60.9	–	99.3	14.6	500	87	45.3	6.3
60-69	1	5	–	81.0	–	342	–	–	–	96.0	25.5	442	80	44.5	9.1
70-79	7	3	1-7	76.6	60.3–90.0	313†	174–427†	66.3†	46.3–75.6						
	1	4	–	69.3	–	242	–	39.2	–	89.0	19.9	354	83	39.1	7.3
80-89	3	3	2-4	55.7	38.6–66.1	266†	155–358†	52.4†	33.5–65.3†	65.3	20.4	289	89	30.8	9.8

*Reported values are rounded off in some cases.
†Indicates PAH (para-aminohippurate).

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wide range of doses for which data are meager, our results are also of interest because they represent a large group of healthy subjects studied under conditions in which complete urine collections were facilitated by continuous observation of the subjects during the period of the collections.

MATERIALS AND METHODS

Subjects were 148 men selected from the Gerontology Ward and Chronic Division of the Baltimore City Hospitals (55 to 92 years old) and from a penal institution in the same geographical area (20 to 39 years old). Subjects from the Gerontology Ward and Chronic Division had been there for several years, primarily for indications which were socioeconomic and associated with advanced age rather than illness. Subjects were selected, after review of all available clinical information, as those who were in good general health. All were ambulatory, free of acute or debilitating chronic illness, were in apparently good nutritional status, and believed to be free of hematologic or gastrointestinal disorder. In no subject was there reason for suspicion of diabetes mellitus. Except for the requirement of urinary continence and the general criteria noted above, there was neither an attempt to select subjects with respect to renal function, nor any clinical indication for suspicion of abnormal function. Status of renal function has been evaluated in 14 of the older subjects as a part of other investigations of the Gerontology Branch on renal function in healthy middle-aged and elderly men. Renal function measurements for these subjects* are given in Table 1 and are seen to be fairly typical for their age groups when compared with the group of healthy subjects studied by Davies and Shock.[19] There was no reason to believe, after review of all available information, that any of the subjects had been administered a source of vitamin B_{12}, except as contained in the institutional diets[20] during the preceding year.

Each subject was given orally (in aqueous solution followed by 2 rinses) 2, 8, 50, or 250 μg of Co^{60}-labeled vitamin B_{12}† (about 2 hours after breakfast and 2 hours before lunch) followed 2 hours after the ingestion of the radioactive vitamin by the intramuscular injection of 1,000 μg of nonradioactive vitamin B_{12}.[11] Following the ingestion of the labeled material, urine was collected for either (1) 0 to 12, 12 to 24, and 24 to 48 hours, or (2) 0 to 24 hours only. When collections at 0 to 12 and 12 to 24 hours were made, results were added to obtain a value for 0- to 24-hour excretion of radioactive vitamin. To facilitate complete urine collection, all subjects were under continuous observation during the collection periods. The procedure used for measurement of the radioactivity excreted into the urine was as previously reported[21] except that the aliquot used for measurement in the present studies was half of that collected. That the excreted radioactivity represents "tagged" vitamin B_{12} has been indicated by chromatographic and other analytic studies,[22] and by differences in storage of radioactivity after $Co^{60}Cl_2$ and Co^{60}-vitamin B_{12} administration.[23]

RESULTS

The results, summarized in Fig. 1, show the effect on the per cent of orally administered radioactive vitamin B_{12} recovered in the urine, of varying (1) the oral dose of $Co^{60}B_{12}$ in amounts of 2, 8, 50, and 250 μg (Fig. 1, *A*, *B*, *C*, and *D*, respectively) and (2) the urine collection intervals (12, 24, and 48 hours) for each of the age groups and the total group.

*We are indebted to Drs. Nathan W. Shock and David Oursler, of the Gerontology Branch, for making available to us the renal function data on subjects included in the present study.

†For supplies of radioactive and nonradioactive vitamin B_{12}, we are indebted to Dr. Charles Rosenblum and Merck & Company, Inc. The present studies were authorized by the Atomic Energy Commission (Authorization Nos. 34126 and 22808), and we gratefully acknowledge the partial support given in a grant-in-aid from the A.E.C. under contract No. AT(30-1) 1203.

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Variations With Age.—The data given in Fig. 1 showed no systematic age difference, for any of the doses or collection periods, in the fraction of dose of cobalt^{60}-vitamin B_{12} excreted in the urine under the experimental conditions of the present study. Further statistical evaluation, in particular for the 24-hour collections when the group aged 20 to 39 years was compared with the group aged 55 to 92 years, revealed no significant age difference: 2 μg, $P > 0.2$; 8 μg, $P > 0.1$; 50 μg, $P > 0.05$; 250 μg, $P > 0.7$. Even when (for 24-hour collections) the group 20 to 39 years old was compared with that 75 years old and older, no consistent age trend was found: 2 μg, $P > 0.1$; 8 μg, $P > 0.01$; 50 μg, $P > 0.6$; 250 μg, $P > 0.2$.

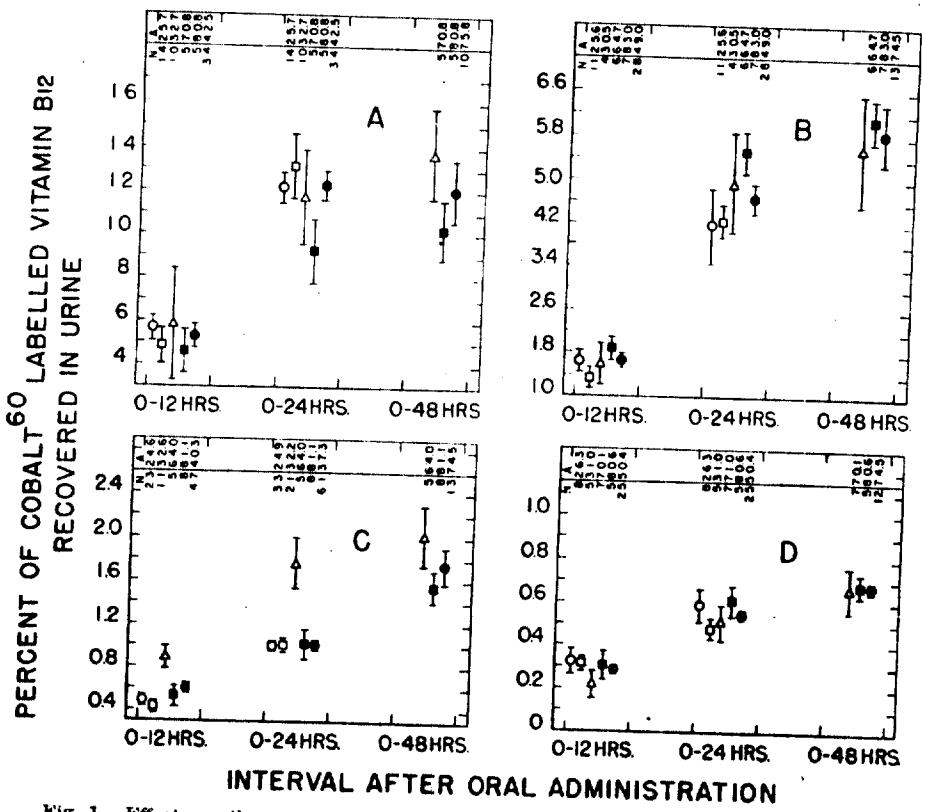

Fig. 1.—Effect, on the per cent of orally administered radioactive vitamin B_{12} recovered in the urine, of varying the oral dose for doses of 2, 8, 50, and 250 μg (A, B, C, and D, respectively), and the urine collection interval (12, 24, and 48 hours) for each of the age subgroups and the combined group of healthy men. Key to symbols: ○ = mean Co^{60}-vitamin B_{12} recovery for subjects of ages 20 to 29 years; □ = mean Co^{60}-vitamin B_{12} recovery for subjects of ages 30 to 39 years; △ = mean Co^{60}-vitamin B_{12} recovery for subjects of ages 55 to 74 years; ■ = mean Co^{60}-vitamin B_{12} recovery for subjects of ages 75 to 92 years; ● = mean Co^{60}-vitamin B_{12} recovery for subjects of all ages combined. Vertical lines define limits of the standard error of the mean. At the top of each diagram is given the number of subjects in each age subgroup (N) and the average age for the subjects in the subgroup (A).

Variations With Collection Interval.—For all of the oral doses considered, the amount of vitamin B_{12} recovered in the 12- to 24-hour urine collection was greater than for the 0- to 12-hour collection. Except for the 2 μg dose, the

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amount of vitamin B_{12} appearing in the collection at 24 to 48 hours was one-fifth to one-half as much as that recovered in the first 24 hours after oral administration. For the 2 μg dose, only small amounts of vitamin B_{12} were recovered in the 24- to 48-hour urine collection.

Variations With Oral Dose.—It is apparent from Fig. 1 that with an increasing amount of vitamin B_{12} administered orally, there was a marked decrease in the *fraction* recovered in the urine, under, of course, the conditions of this study. As the oral dose was increased, however, the calculated *amount* of vitamin B_{12} recovered in the urine definitely increased, as shown by the mean values and standard deviations in Table II.

DISCUSSION

A. Effect of Parenteral Dose and Tissue Retention on Results.—The urinary excretion of orally administered vitamin B_{12} in the present study represents only a minimum value for the amount absorbed. The observed difference between the amount apparently absorbed and that excreted in the urine[12] is due, to some extent, to the parenteral dose, since this has been observed to decrease the proportion of an oral dose absorbed from the gastrointestinal tract.[12, 24] The effect of the parenteral dose, however, would seem to be small compared to that due to tissue retention; a large proportion of the absorbed material is retained in the liver.[5, 25] From these considerations and the results of the present study, it would appear that the average amount of vitamin B_{12} absorbed from a single 250 μg dose probably greatly exceeds 1.7 μg, since the latter was the average amount of labeled vitamin B_{12} recovered in the urine within 48 hours after the administration of 250 μg of labeled material.

B. Relation of Amount and Time of Vitamin B_{12} Absorption to Oral Dose.—The character of the relationship obtained in the present study, between oral dose and the amount of vitamin B_{12} excreted in the urine, seems to be in remarkably good agreement with that indicated by the results of Glass and associates.[25] These workers, using the hepatic uptake technique and oral dose range of 0.5 to 50 μg, noted increase in amount and decrease in fraction taken up by the liver in subjects given larger oral doses compared to subjects given smaller doses of labeled vitamin B_{12}. While in agreement with this work, our findings differ from the conclusions of Callender and Evans[1] and Swenseid and co-workers,[26] who used the fecal excretion technique and found optimal absorption at doses not exceeding about 2 and 5 μg, respectively. It seems conceivable that the "plateau" in absorption observed by the latter 2 groups was due to interference associated with successive tests in the same subjects.

The distributions of radioactivity between the several urine collections, in the present study, are consistent with the view that vitamin B_{12} absorption proceeds gradually, and approaches maximal levels sooner for smaller oral doses.

The relationship of the fraction and amount of vitamin B_{12} recovered in the urine to the oral dose of the vitamin are consistent with the views that

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TABLE II. AMOUNTS OF VITAMIN B_{12} RECOVERED IN THE URINE FOR VARIOUS ORAL DOSES, FOLLOWED BY A PARENTERAL DOSE, IN APPARENTLY HEALTHY MEN OF AGES 20 TO 92 YEARS

ORAL DOSE (μg)	RECOVERIES OF VITAMIN B_{12} IN URINE (MICROGRAMS)											
	0 TO 12 HOURS				0 TO 24 HOURS				0 TO 48 HOURS			
	N*	RANGE	MEAN	S.D.†	N	RANGE	MEAN	S.D.	N	RANGE	MEAN	S.D.
2	34	0.002-0.28	0.11	0.06	34	0.09-0.48	0.24	0.08	10	0.11-0.41	0.24	0.09
8	28	0.008-0.23	0.13	0.06	28	0.13-0.70	0.37	0.11	13	0.21-0.80	0.46	0.16
50	47	0.06-0.77	0.25	0.14	61	0.33-1.22	0.55	0.17	13	0.45-1.30	0.84	0.30
250	25	0.30-1.65	0.70	0.20	25	0.63-2.53	1.35	0.24	12	0.68-2.80	1.66	0.21

*N = number of subjects.
†S.D. = standard deviation of distribution.

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there are at least 2 mechanisms of absorption of vitamin B_{12} from the gastrointestinal tract* in normal people, that these mechanisms act concurrently, and that at different oral dose levels the 2 mechanisms are of relatively different importance. Thus, at low (physiologic) dose levels, the available intrinsic factor is of relatively great importance in influencing the amount absorbed, while at the higher dose levels intrinsic factor is of decreased relative importance and the amount absorbed seems to be more directly related to the amount administered, i.e., a "mass" effect. Although it has not yet been clearly demonstrated to mediate vitamin B_{12} absorption, diffusion would seem to be the simplest mechanism consistent with existing observations of absorption apparently beyond that mediated by intrinsic factor, insofar as these data have been analyzed. It would seem reasonable to suppose that absorption by this mechanism would be negligible or, at least, inadequate to meet nutritional requirements, at low (physiologic) dose levels. At usual dietary levels, then, adequate amounts of the vitamin would be expected to traverse the intestinal mucosa only if adequate amounts of intrinsic factor were available. The manner by which this traversal comes about (at low and high oral dose levels), while the subject of conceptually appealing speculations[25, 27] with which our results are not inconsistent, will remain speculative until many more data have become available and adequately interpreted.

C. Age Differences.—

1. Effect on results of age differences in renal function: In consideration of the possible effect of renal function on the results, it should be noted that subjects included in the present study probably have renal function rather typical for healthy subjects in their respective age groups (see Introduction and Table I). Even for healthy men, however, there is regression of renal function on age.[19] In the absence of such influence in our older subjects, however, the values for excretion of vitamin B_{12} might have been *higher* but not lower than those we observed. Had it happened that significantly less radioactive vitamin B_{12} had been excreted by the older, as compared to the younger, subjects, then the difference might possibly have been explained on the basis of lower renal function in the older subjects. Such did not occur, however, and the fact that the urine collections for the older men contained as much radioactivity as did those for the young subjects cannot be explained on the basis of age differences in renal function.

Failure to observe decreased excretion of Co^{60}-vitamin B_{12} in the older subjects we have studied was not likely to have been due to decreased tissue retention of absorbed vitamin B_{12}, in the old as compared to the young men, in view of the following considerations: (1) amounts of radioactive vitamin B_{12} retained by the liver after oral administration were found by Glass and coworkers[6] to be not significantly different in old and young men; (2) the large parenteral dose of vitamin B_{12}, given to all subjects, would be expected to

*Since this paper was submitted, there has appeared a paper by Doscherholmen and Hagen which supports the existence of a dual mechanism in the absorption of vitamin B_{12}. (Doscherholmen, A., and Hagen, P. S.: A Dual Mechanism of Vitamin B_{12} Plasma Absorption, J. Clin. Invest. 36: 1551-1557, 1957.)

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give a higher degree of tissue saturation with the vitamin (before the radioactive vitamin entered the plasma) than would otherwise have been the case; (3) retention of parenterally administered doses of 20 to 75 μg of vitamin B_{12} is probably even greater in old men than in young men.[28]

2. Comparison of our results with those of other studies related to absorption of vitamin B_{12} and aging: Chow and associates[21] measured the urinary excretion of radioactive vitamin B_{12} following oral administration of 2 μg of labeled material and a large parenteral dose; in 40 healthy subjects 19 to 85 years old, no significant age difference was observed. Using the hepatic uptake method and a test dose of 0.5 μg of vitamin B_{12}, Glass and co-workers[6] found no significant age difference in radioactive vitamin B_{12} uptake by the liver in 60 subjects aged 18 to 90 years who were normal or had some irrelevant disorder. In other reports, the number of "healthy" or "control" subjects has been inadequate for statistical treatment of the data for agewise study (8 "controls" in Schilling's[29] paper, for example) or adequate information on ages of subjects has been omitted.[12]

3. Relation of the present study to evidence for age differences in serum vitamin B_{12} levels: Recent studies in healthy human subjects in our laboratories[20] and elsewhere[30, 31] have indicated that there is a decrease in serum concentration of vitamin B_{12} microbiologic activity with advancing age. The results of the present study for doses of 2 to 250 μg, other studies[21] for the 2 μg dose, and studies of hepatic uptake for the 0.5 μg dose[6] do not support the contention that age differences in serum levels of vitamin B_{12} are due to impairment of absorption of the vitamin. It is possible, although not yet clearly demonstrated, that the lower levels of serum vitamin B_{12} observed in the aged subjects may have been associated with decreased ingestion of vitamin B_{12} due to dietary selection by the subjects themselves,[20] in the absence of any significant impairment in vitamin B_{12} absorption. It should be emphasized, however, that tests using oral doses of 2 μg and larger, such as in the present study, are not representative of absorption for the smaller doses in which the best separations have been found between patients with intrinsic factor deficiency and normal subjects.[4, 13, 18] Smaller doses, moreover, would seem to closely approach levels more frequently found in the diet (and are, in this sense, physiologic doses). The data do suggest, however, that amounts of vitamin B_{12} absorbed from oral doses in the 2 to 250 μg range are not significantly less in old men than in young men.

SUMMARY

1. Labeled material appearing in the urine in 12, 24, and 48 hours after oral administration of 2, 8, 50, or 250 μg of cobalt^{60}-labeled vitamin B_{12} (cyanocobalamin or derivative), followed after 2 hours by intramuscular injection of 1,000 μg of nonradioactive vitamin B_{12}, has been estimated in 148 apparently healthy men 20 to 92 years old.

2. With increasing oral dose, for the range considered, there was observed marked decrease in *fraction* but considerable increase in *amount* of vitamin

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B_{12} appearing in the urine. For all doses, the amount of vitamin B_{12} recovered in the 12- to 24-hour urine collection was greater than for the collection at 0 to 12 hours. Except for the 12 μg dose, the amount of vitamin B_{12} appearing in the 24- to 48-hour collection was one-fifth to one-half as much as that recovered in the first 24 hours after oral administration. Mean recoveries in micrograms of labeled vitamin B_{12} in the urine ± standard deviation of distribution for each of the oral doses and for 0 to 12, 0 to 24, and 0 to 48 hours after oral administration, respectively, were: (1) *2 μg*: 0.11 ± 0.06, 0.24 ± 0.08, 0.24 ± 0.09; (2) *8 μg*: 0.13 ± 0.06, 0.37 ± 0.11, 0.46 ± 0.16; (3) *50 μg*: 0.25 ± 0.14, 0.55 ± 0.17, 0.84 ± 0.31; (4) *250 μg*: 0.70 ± 0.20, 1.35 ± 0.24, 1.66 ± 0.21.

3. The data are consistent with the views that there are at least two mechanisms of absorption of vitamin B_{12} from the gastrointestinal tract, that these mechanisms act concurrently and have relatively different importance at different dose levels, and that absorption proceeds gradually, approaching maximal levels sooner for smaller dose levels.

4. Age differences in urinary excretion or orally administered material were not demonstrated for any of the doses under the experimental conditions of the study. The oral doses used, however, were higher than those for which best separation has been demonstrated between normal subjects and patients with intrinsic factor deficiency. The excreted material probably represents that absorbed, to some extent for the smallest dose and in large proportion for the larger doses, by a mechanism relatively independent of the amount of intrinsic factor available. The efficiency of such a mechanism, insofar as indicated by the urinary excretion test, was not impaired in the older, as compared to the younger, subjects.

We wish to express our gratitude to many individuals of the staffs of the Gerontology Branch, Baltimore City Hospitals and the Department of Biochemistry, Johns Hopkins School of Hygiene and Public Health, for their aid in the present study, including technical assistance and reading of the manuscript; to the Nursing Staff of the Baltimore City Hospitals for observation of subjects during the periods of urine collections; and to Drs. Patricia McIntyre and Julius Krevans for their interest in the work.

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Vitamin B_{12} Uptake by Intestinal Microorganisms: Mechanism and Relevance to Syndromes of Intestinal Bacterial Overgrowth

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ABSTRACT The mechanism of bacterial uptake of vitamin B_{12}, the spectrum of microorganisms capable of such uptake, and the factors involved were the subject of this study. Bacterial uptake of vitamin B_{12} was found to be at least a two stage process. A primary uptake phase which was rapid (1 min or less), pH dependent, nontemperature dependent, did not require viable organisms and was insensitive to either the metabolic inhibitor dinitrophenol or to the sulfhydryl inhibitor *N*-ethyl-maleimide. Protein denaturation (formalin treatment or autoclaving) abolished all B_{12} uptake. This primary uptake phase is thought to represent adsorption to binding or "receptor" sites on the cell wall. Second stage uptake was slower, pH and temperature dependent, required living bacteria, and was abolished by either dinitrophenol or *N*-ethyl-maleimide. This phase is dependent upon metabolic processes and may reflect transfer of B_{12} from surface "receptor" sites into the bacterial cell. Although differences among organisms were observed in total 1 hr uptake, number of surface "receptor" sites, and relative avidities for B_{12}, all organisms except *Streptococcus fecalis* shared the two stage mechanism. Two Gram-positive organisms, *Bacillus subtilis* and Group A streptococcus, demonstrated the highest 1 hr vitamin B_{12} uptake values; Gram-negative bacteria required 2,000–10,000 the number of organisms for comparable uptake. Binding constants (K_m) varied from $5.05 \pm 1.67 \times 10^{-10}$M for *B. subtilis* to $6.18 \pm 3.08 \times 10^{-9}$M for *Klebsiella pneumoniae* which approximate the *Km* for human intrinsic factor (0.38×10^{-10}M). Competition between bacteria and intrinsic factor for vitamin B_{12} may be inferred from the similarity of these constants.

These observations suggest that a variety of enteric and nonenteric organisms, not requiring exogenous B_{12}, may play a role in the pathogenesis of the vitamin B_{12} malabsorption found in the intestinal bacterial overgrowth syndromes.

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INTRODUCTION

In recent years, attention has been focused upon the relationship of bacterial uptake of vitamin B_{12} to the vitamin B_{12} malabsorption present in patients with intestinal bacterial overgrowth (1–5). Donaldson (1) and Donaldson, Corrigan, and Natsios (2), demonstrated that bacteria isolated from surgically created blind loops in rats could take up significant amounts of vitamin B_{12} which had been previously bound to I.F. (Intrinsic Factor). It is not clear, however, whether the entire I.F.–B_{12} complex was taken up or whether the B_{12} was detached from I.F. by the bacteria before or during the uptake process. While these studies have contributed to our understanding of the interrelationships between bacteria and vitamin B_{12}, the mechanism of B_{12} uptake by bacteria, the spectrum of bacteria capable of B_{12} uptake, and factors determining such uptake remain unclear. The present studies are concerned with these points and demonstrate that a wide variety of nonvitamin B_{12} requiring enteric and nonenteric bacteria take up vitamin B_{12} by a two stage process.

METHODS

Escherichia coli K_{12} (wild type) was the prototype organism, (hereafter referred to as *E. coli*). Other organisms

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used were obtained from the stock culture collection of the Department of Microbiology, Boston University School of Medicine.[1] Cyanocobalamine[2] labeled with ^{57}Co (specific activity 13–18 mCi/mg) was used in all experiments and was diluted with nonradioactive cyanocobalamine[2] to give the desired B_{12} concentrations.

All procedures were carried out with aseptic technique. The various solutions used were sterilized by filtration through a Millipore filter with a pore size of 0.45 μ.[3]

Growth and preparation of cells. Organisms were stored at 5°C on trypticase soy agar[4] slants until used. An 18 hr growth (in trypticase soy broth[4] with a vitamin B_{12} concentration of less than 0.005 ng/ml) of each organism was used throughout. This consistently gave cultures in early stationary growth phase as demonstrated by sequential culturing. The coefficient of variation (6) on day to day determinations was 9%. Organisms were quantitated in duplicate by the serial dilution and drop plate technique (7). Replicate plating of a single culture of various organisms yielded a coefficient of variation of 15–17%. When plate counts were compared with direct microscopic counts, the results were the same. Thus, nonviable organisms did not comprise a significant portion of the bacterial population.

Organisms were harvested by centrifugation for 30 min at 3500 rpm, the bacterial sediment washed with sterile saline, recentrifuged, and suspended in a volume of distilled water to obtain the desired number of bacteria as determined turbidimetrically. This bacterial suspension was used immediately and the number or bacteria quantitated for each experiment.

Unless otherwise specified, each tube contained a total of 4 ml (pH 6.9–7.1) consisting of 1 ml of each of the following: (*a*) bacterial suspension, (*b*) 0.6 ng ^{57}Co vitamin B_{12}/ml, and 2 ml of isotonic saline. In studies concerned with the effects of the inhibitors or pH, 2 ml of the appropriate aqueous solution of inhibitor of buffer was substituted for isotonic saline. Each determination was done in duplicate.

Measurement of bacterial uptake of vitamin B_{12}. A modification of the microbial adsorption inhibition method of Grasbeck (8) was used as follows: bacteria were incubated at 37°C for various periods of time with vitamin B_{12}-^{57}Co and were separated from the rest of the reaction mixture by centrifugation. The radioactivity of each tube was counted before organisms were added and the total radioactivity remaining in the supernatant determined by counting a 1 ml portion. Per cent vitamin B_{12} uptake was calculated by dividing the amount of radioactivity removed from the supernatant by the initial total radioactivity. In some studies the bacterial pellet was washed, digested overnight with concentrated H_2SO_4, and the radioactivity bound to the bacteria thus directly measured. Both procedures gave comparable results. A Packard gamma isotope counter (Packard Instrument Co., Downers Grove, Ill.) with a counting error of 0.5–1.0% was used. Recovery of added radioactivity ranged between 95 and 105%.

At the conclusion of each experiment the pH of the supernatant was measured with a glass electrode, the bacterial pellet was resuspended in distilled water to a volume of 1 ml and the number of surviving bacteria counted by the serial dilution and drop plate technique (7). pH never varied more than 0.2 pH units and the number of bacteria recovered was identical with the number originally added.

Results are expressed as mean ±1 SE and were analyzed for statistical significance by the Student t test (6).

RESULTS

Accuracy of the method

The accuracy and reproducibility of this method was determined in two ways. In eight replicate samples the 1 hr vitamin B_{12} uptake by (mean ±SE) *E. coli* (1×10^9 organisms) was 96.9 ±0.03%; and of 52 samples done on 26 different days 92.0 ±0.05%.

Kinetics of bacterial uptake of vitamin B_{12}; demonstration of two stage process

Bacterial uptake of vitamin B_{12} with time. The velocity of the reaction between *E. coli* and vitamin B_{12} is shown in Fig. 1. Two distinct components were noted in the uptake curve: an initial rapid stage (1 min or less) and a slower second stage. The 1 min uptake value is an approximation of the initial rate of uptake since it was not feasible to measure B_{12} uptake in less than 1 min with the methods used. Both the first stage uptake and total 1 hr uptake (which includes both first and second stage) increased with increasing numbers of *E. coli*.

Effect of vitamin B_{12} concentration on first stage uptake. When a fixed number of bacteria was incubated with increasing concentrations of vitamin B_{12}, 1 min (first stage) bacterial uptake of vitamin B_{12} demonstrated saturation kinetics. These data were analyzed by the method of Lineweaver and Burk (9) and a *Km* for first stage B_{12} uptake was calculated (Table I). For *E. coli*, the *Km* was $6.03 \pm 0.62 \times 10^{-10}$M (mean ±1 SE). Additional *Km* values for various aerobic bacteria, also listed in Table I, illustrate the similarity among 10 dif-

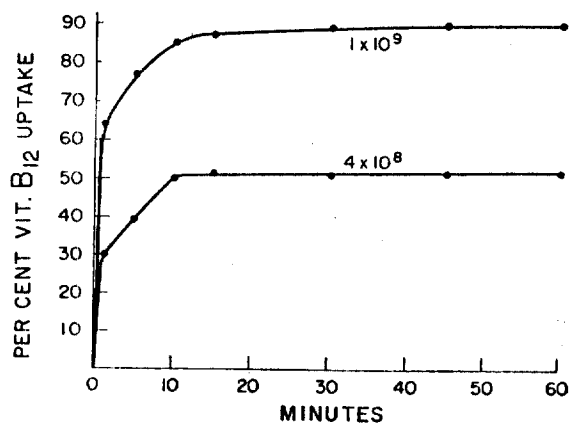

FIGURE 1 Vitamin B_{12} uptake by various numbers of *E. coli*. Typical plot representative of eight experiments. Bacteria were incubated with 0.6 ng vitamin B_{12} at 37°C and at pH 7.0 as described in text.

[1] Kindly supplied by Dr. E. E. Baker and Dr. L. M. Corwin.

[2] E. R. Squibb & Sons, Division of Olin Mathieson Chem. Corp., New York.

[3] Millipore Corp., Bedford, Mass.

[4] Baltimore Biological Laboratory, Baltimore, Md.

TABLE I

*K_m (Binding Constant) Values of Aerobic Bacteria**

‡A. *Bacillus subtilis*	$5.05 \pm 1.67 \times 10^{-10}$ M
§*Escherichia coli* K_{12} (mutant)	$5.38 \pm 1.63 \times 10^{-10}$ M
Escherichia coli K_{12} (wild type)	$6.03 \pm 0.62 \times 10^{-10}$ M
B. *Proteus mirabilis*	$8.34 \pm 1.77 \times 10^{-10}$ M
Escherichia coli B	$1.08 \pm 0.26 \times 10^{-9}$ M
Salmonella paratyphi	$2.00 \pm 0.74 \times 10^{-9}$ M
C. *Pseudomonas aeruginosa*	$2.00 \pm 0.48 \times 10^{-9}$ M
Staphylococcus aureus	$3.15 \pm 0.33 \times 10^{-9}$ M
Group A streptococcus (β-hemolytic)	$3.16 \pm 0.18 \times 10^{-9}$ M
Klebsiella pneumoniae	$6.18 \pm 3.08 \times 10^{-9}$ M

* Values expressed as mean ±SE.
‡ Bacteria within each group not significantly different from each other $P = > 0.05$. Groups A and C significantly different from each other $P = < 0.05$, but neither group significantly different from group B. $P = > 0.05$.
§ Methionine requiring mutant.

TABLE II

*Saturation of First Stage Bacterial Uptake of Vitamin B_{12}**

Bacteria‡	Saturating amount ng/10^9 organisms§	Molecules vit. B_{12} bound/organism
1. Group A. streptococcus (4)	799.8 ±117.5	355,000‖
2. B. subtilis (5)	402.4 ±69.8	179,000
3. *Kleb. pneumoniae* (4)	15.0 ±4.6	6,680
4. *Ps. aeruginosa* (5)	5.0 ±0.4	2,230
5. *Sal. paratyphi* (6)	4.7 ±1.1	2,090
6. *P. mirabilis* (4)	3.5 ±1.2	1,550
7. *E. coli* K_{12} (wild type) (6)	2.9 ±0.4	1,280
8. *Staph. aureus* (4)	2.6 ±0.7	1,140
9. *E. coli* B (4)	2.2 ±0.3	950
¶10. *E. coli* K_{12} (mutant) (6)	2.2 ±0.5	950
11. *Strep. fecalis* (5)	0	0

* Group A streptococcus and *B. subtilis* significantly different from each other and from all others $P = < 0.05$. *Kleb. pneumoniae* and *Ps. aeruginosa* not significantly different from each other, $P = > 0.05$, but each is significantly different from organisms 7–11, $P = < 0.05$. Organisms 5–10 are not significantly different, $P = > 0.05$.
‡ Numbers in parentheses represent number of trials.
§ Expressed as mean ±SE.
‖ Calculated from saturating amount

(No. moles B_{12} × Avogadro's No.)/(No. bacteria)

When data calculated by the method of Scatchard (10), values obtained were essentially the same as those listed.
¶ Methionine requiring mutant.

ferent bacteria, with *Bacillus subtilis* having the smallest *Km* and *Kleb. pneumoniae* the largest. There were no significant differences in the *Km* values among three different strains of *E. coli*.

First stage (1 min) B_{12} uptake by *E. coli* (1×10^9 organisms) was saturated by 2.9 ±0.4 ng of vitamin B_{12}. The quantity of B_{12} necessary for first stage saturation of a variety of organisms is listed in Table II. The last column of Table II shows the number of molecules of B_{12} necessary for first stage saturation. The wide range in the number of B_{12} molecules bound at saturation of first stage is apparent (355,000 for Group A streptococcus to 950 for *E. coli* K_{12} [mutant]). *Strep. fecalis* was the sole exception demonstrating no significant B_{12} uptake. There was no correlation between the amount of B_{12} bound at saturation and *Km* value.

1 hr bacterial uptake of vitamin B_{12}. As shown in Table III, B_{12} uptake by *E. coli* (1×10^9) and *Salmonella paratyphi* (1×10^9) after 1 hr was 7.0 and 10.7 ng respectively. Gram-negative enteric organisms varied between 6.6 ±1.0 and 255 ±35.9 ng/hour and Gram-positive organisms, between 3.2 ±0.9 and 21,700 ±2200 ng/hour. All organisms demonstrated saturation kinetics. *Strep. fecalis*, however, demonstrated no significant B_{12} uptake in 1 hr.

TABLE III

*1 Hr Vitamin B_{12} Uptake of Aerobic Bacteria**

Bacteria‡	1 hr uptake§
	ng
1) *B. subtilis* (6)	21,700 ±2,200
2) Group A streptococcus (7)	1,810 ±258
3) *P. mirabilis* (5)	255 ±35.9
4) *E. coli* B (6)	30.6 ±6.7
5) *Sal. paratyphi* (5)	19.7 ±4.1
6) *Kleb. pneumoniae* (5)	17.3 ±7.8
7) *Ps. aeruginosa* (4)	12.5 ±2.9
8) *E. coli* K_{12} (wild type) (5)	7.0 ±1.5
‖9) *E. coli* K_{12} (mutant) (12)	6.6 ±1.0
10) *Staph. aureus* (5)	3.2 ±0.9
11) *Strep. fecalis* (5)	0

* Number of bacteria in each case 1×10^9.
‡ Numbers in parentheses represent number of trials.
§ Values expressed as mean ±1 SE. The only significant differences, $P = < 0.05$, as follows: *B. subtilis*, Group A. streptococcus, and *P. mirabilis* from each other and all others; *E. coli B* from organisms 7–11; *Sal. paratyphi* from organisms 9–11; *Kleb. pneumoniae* from *Strep. fecalis*; *Ps. aeruginosa* from *Staph. aureus* and *Strep. fecalis*; *E. coli* K_{12} (wild type) from *Strep. fecalis*; *E. coli* K_{12} (mutant) from *Staph. aureus* and *Strep. fecalis*; and *Staph. aureus* from *Strep. fecalis*.
‖ Methionine requiring mutant.

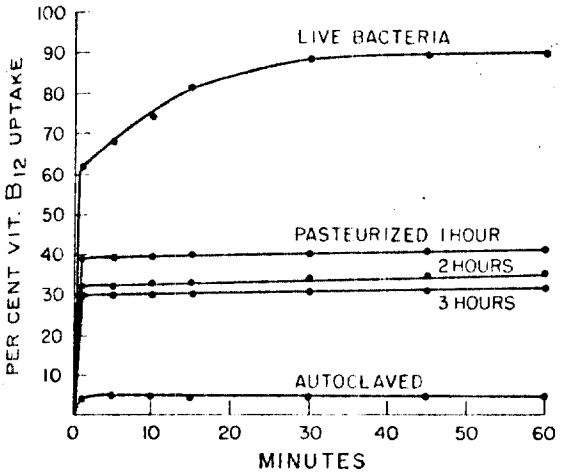

FIGURE 2 Effect of bacterial viability on vitamin B_{12} uptake. *E. coli*, live and killed as indicated, were incubated with 0.6 ng vitamin B_{12} at 37°C and at pH 7.0 as described in text. Bacteria were pasteurized by incubation in a water bath at 60°C for the times indicated. Bacteria were autoclaved at 120°C for 20 min. Number of bacteria initially 1×10^9 in each instance. Plate counts of pasteurized and autoclaved bacteria revealed no surviving bacteria. Fig. 2 is typical of three experiments.

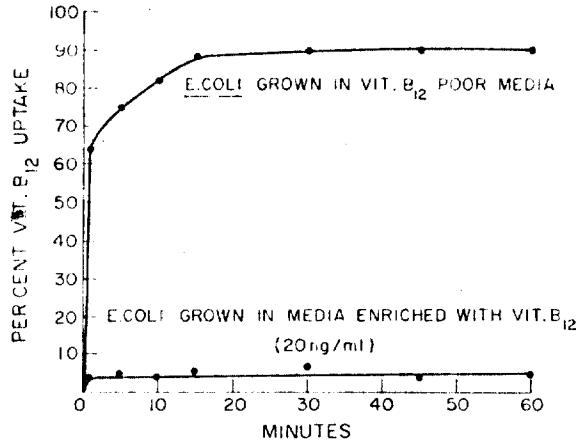

FIGURE 3 Effect of prior vitamin B_{12} exposure on vitamin B_{12} uptake by *E. coli*. Bacteria were grown in vitamin B_{12} poor media or in media enriched with nonradioactive vitamin B_{12} (20 ng/ml). Bacteria were harvested and prepared as described in text and incubated with 0.6 ng B_{12}-^{57}Co at 37°C and at pH 6.9. Number of bacteria in each instance 1×10^9. This fig. is typical of three trials.

Effect of viability of bacteria on vitamin B_{12} uptake

E. coli autoclaved at 120°C for 20 min showed no vitamin B_{12} uptake (Fig. 2). Coincident with cellular disruption and protein denaturation, Gram-staining reaction was also lost. *E. coli*, pasteurized at 56°C for 1–3 hr, retained approximately one-half the control first stage vitamin B_{12} uptake but little second stage uptake. Although viability was lost, bacteria appeared intact microscopically, and retained their Gram-staining properties.

Effect of prior vitamin B_{12} deprivation or saturation on subsequent bacterial uptake of vitamin B_{12}

In these experiments, *E. coli* was grown in trypticase soy broth either with or without added nonradioactive vitamin B_{12} (20 ng/ml). After 24 hr the bacteria were counted (no difference was noted in number between those grown in the presence or absence of B_{12}), harvested by centrifugation, washed three times with 0.15 M saline and an aqueous suspension prepared as described in Methods.

The B_{12} uptake rate of 1×10^9 of each *E. coli* was then measured. As shown in Fig. 3, *E. coli* grown in media enriched with nonradioactive vitamin B_{12} lost its ability to take up radioactive vitamin B_{12}, as compared to organisms grown in media lacking vitamin B_{12}.

Effect of temperature on vitamin B_{12} uptake

First stage uptake (Fig. 4) was largely independent of temperature while second stage uptake was clearly temperature dependent. At 5°C the second stage uptake rate was substantially reduced such that the total 1 hr uptake was only 13% greater than 1 min uptake. At 37°C second stage uptake was 50% greater.

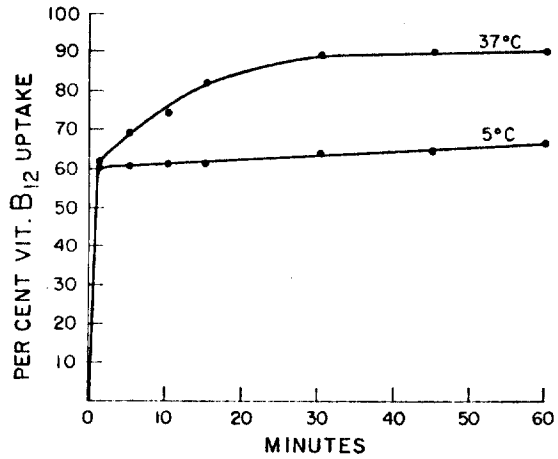

FIGURE 4 Effect of temperature on bacterial uptake of vitamin B_{12}. *E. coli* (1×10^9) incubated with 0.6 ng vitamin B_{12} at either 5°C or 37°C at pH 7.1 as described in text. Fig. 4 is typical of five trials.

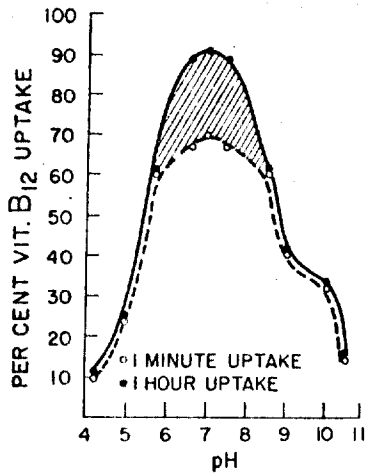

FIGURE 5 Effect of pH on vitamin B_{12} uptake by *E. coli*. Per cent vitamin B_{12} uptake at 1 min and 1 hr plotted against pH. The difference between 1 min and 1 hr (shaded area) represents second stage uptake. Bacteria (1×10^9) were incubated with 0.6 ng vitamin B_{12} at 37°C at the indicated pH as described in the text. pH 4.2, 5.0, and 5.7 maintained with 2 ml 0.1 M citrate buffer; pH 6.6, 7.0, and 7.5 with 2 ml 0.2 M phosphate buffer; and pH 8.5, 9.0, 10.0, and 10.5 with 2 ml 0.2 M bicarbonate buffer. Various buffers of same pH gave comparable values. The number of bacteria surviving at the conclusion of each experiment was unchanged from the number initially added. Fig. 5 is typical of three experiments.

TABLE IV

*Effect of Inhibitors on E. coli Vitamin B_{12} Uptake**

		1 min uptake %	60 min uptake %
Control		60	90
Formalin	0.30%	58	74
	2.5%	36	52
	10.0%	2	12
Dinitrophenol	0.025 mM	60	80
	0.20 mM	58	61
	0.50 mM	59	60
	1.0 mM	60	59
N-ethyl-maleimide	10^{-7} M	60	72
	10^{-4} M	60	71
	10^{-2} M	56	65
NaF	2.5 mM	60	90
	25.0 mM	59	91
	100.0 mM	61	89
Acetylstrophanthin	10^{-8} M	60	90
	10^{-5} M	59	89
	10^{-2} M	61	89

* Figures represent mean values of three experiments per inhibitor.

Effect of pH on bacterial uptake of vitamin B_{12}

For both first and second stage vitamin B_{12} uptake, optimum was between 6.6 and 7.5. As shown in Fig. 5, both stages of B_{12} uptake were pH sensitive, with second stage uptake being more sensitive to pH change. As the pH was varied from the optimum range first stage uptake progressively decreased, but was never completely abolished. Second stage uptake, however, was completely abolished as the pH was either lowered or elevated out of the optimum range. The number of bacteria surviving at the conclusion of each experiment was unchanged from the number initially added.

Effect of various inhibitors on bacterial uptake of vitamin B_{12}

Increasing concentrations of formalin progressively inhibited both first and second stage *E. coli* vitamin B_{12} uptake (Table IV). Dinitrophenol and *N*-ethyl-maleimide selectively inhibited only second stage B_{12} uptake, while sodium fluoride and acetylstrophanthin were without effect on either stage.

Number of bacteria necessary for vitamin B_{12} uptake

Approximately 2×10^7 *E. coli* were necessary before vitamin B_{12} uptake could be detected and 1×10^9 organisms were necessary to give 90% uptake constantly. Other Gram-negative rods, i.e. *Proteus mirabilis*, *Salmonella paratyphi*, *Pseudomonas aeruginosa*, and *Kleb. pneumoniae*, were similar to *E. coli*, but Gram-positive organisms demonstrated a greater spectrum. Group A streptococcus and *B. subtilis* showed appreciable B_{12} uptake with as few as 5×10^4 and 6×10^5 organisms respectively. *Staphylococcus aureus* was similar to the Gram-negatives while *Strep. fecalis* was unable to take up significant amounts of B_{12} even with numbers as high as 1×10^{11} organisms.

Other bacteria demonstrating vitamin B_{12} uptake

Other Gram-positive and negative bacteria tested which demonstrated B_{12} uptake included *Aerobacter aerogenes*, *Shigella sonnei*, and *Shigella flexneri*, *Salmonella typhimurium* and *Salmonella schottmuelleri*, *Corynebacterium diphtheriae*, three strains of Group A streptococci, and two strains each of Group C and G streptococci. All organisms studied, with the exception of *Strep. fecalis* (see above), demonstrated B_{12} uptake and had a similar two stage vitamin B_{12} uptake process.

DISCUSSION

These studies, concerned with the mechanism of vitamin B_{12} uptake by bacteria, indicate that B_{12} is taken up by a variety of microorganisms, which do not require ex-

ogenous B_{12}. This process which occurs in at least two stages, is similar to that in the vitamin B_{12} requiring parasite *Ochromonas malhamensis* (11) and various B_{12} requiring species of lactobacillus (12).

While a precise delineation of the mechanisms involved is not possible at present, certain conclusions may be drawn from the available data. Simple diffusion through cell membranes is not likely owing to the physical size and hydrophilic properties of vitamin B_{12}. That binding sites function either as the first step of a carrier system or as a step before transport into the cell appears more likely. This interpretation is compatible with the following data:

First stage uptake. First stage uptake (1 min or less) was nontemperature dependent, insensitive to metabolic inhibitors, and occurred (though to a lesser degree) even when bacteria were killed by pasteurization. Thus, viability of bacteria per se, is not a necessary prerequisite for first stage B_{12} uptake. However, since pasteurization decreased first stage uptake by 50%, it is likely that some of the surface B_{12} binding sites were denatured by pasteurization (13). With more drastic heat treatment, i.e. autoclaving, the bacterial cells were disrupted, and all B_{12} uptake and Gram-staining reaction abolished. Formalin inhibition of B_{12} uptake may also be related to protein denaturation. Formalin, which reacts with amino, imino, hydroxyl, and many other groups to form methylene bridges between proteins (14), presumably alters the surface B_{12}-binding sites.

Data in support of the concept of cell wall B_{12} binding sites have been presented by Sasaki and Kitahara (15) who fractionated *Lactobacillus leichmannii* after 30 min of radioactive vitamin B_{12} uptake and found 55% of the radioactivity in the cell wall fraction. Clues to the nature of the surface binding sites are provided by the observations that the vitamin B_{12} could not be washed off, nor could it be removed when cell walls were subjected to digestion by trypsin, RNAase, or pepsin (15). Failure to inhibit first stage uptake by the sulfhydryl inhibitor N-ethyl-maleimide indicates that —SH groups are not involved in this binding. pH, however, is of major import. At pH less than 5.7 or greater than 7.5, B_{12} uptake was progressively decreased, a finding noted also by Davis and Chow (16). This suggests the possibility of dissociation of binding sites on the bacterial cell wall, similar to that postulated for the parasite *Ochromonas* (11). Since bacterial surfaces possess a net negative charge (most likely due to dissociated acid groups [17]), sensitivity to both high and low pH is compatible with a binding phenomenon of electrochemical nature.

First stage uptake was directly proportional to the number of bacteria. Preloading with vitamin B_{12} abolished further B_{12} uptake, presumably by saturating both the surface binding sites and intracellular B_{12} storage capacity. While this interpretation is reasonable, low radioactive B_{12} uptake values could, in part, reflect dilution of the radioactive B_{12} by cold B_{12} exiting from the bacteria (12). Nevertheless, the fact that first stage uptake exhibited saturation kinetics is evidence for the existence of binding or "receptor" sites. The wide range observed in the number of B_{12} molecules bound per organisms (950–355,000) at saturating concentrations, also point out the wide range in the number of binding sites among the organisms studied. Similar data for various B_{12} requiring organisms have been reported, i.e., *Lactobacillus leichmannii* 10,500 molecules/organism (12), *E. coli* 1,150/organism (18), and *Ochromonas malhamensis* 524,000/organism (19).

Application of Michaelis-Menton enzyme-substrate kinetic analysis to bacterial transport systems has been made with a variety of substrates (20, 21). Such analysis permits calculation of *Km*, which for the purpose of this study is an affinity or binding constant, for first stage B_{12} uptake. As with enzyme-substrate interactions, the smaller the *Km* value the greater the avidity of bacteria for vitamin B_{12}. *Km* values in the present studies ($6.03 \times 10^{-10} - 6.18 \times 10^{-8}$M) show great avidity of bacteria for B_{12} and are similar to the *Km* reported for *Lactobacillus leichmannii* (2.8×10^{-9}M) by Kashket, Kaufman, and Beck (12) and approximates the *Km* reported for human intrinsic factor (0.38×10^{-10}) by McGuigan (22).

It is concluded that the first stage B_{12} uptake by bacteria is an adsorptive process related to vitamin B_{12} binding or "receptor" sites on the cell wall.

Second stage uptake. Second stage bacterial uptake of vitamin B_{12} was pH and temperature dependent, required living bacteria, and was inhibited by dinitrophenol and *N*-ethylmaleimide. While this seems to be a metabolic process, second stage uptake was insensitive to NaF or acetylstrophanthin, suggesting that neither glycolysis nor Na-K activated membrane bound adenosine triphosphatase (ATPase) appeared to be involved. Supporting the concept that second stage B_{12} uptake may represent transport of vitamin B_{12} from the cell wall receptor sites into the cell interior, are the studies of Kashket, Tave, and Beck (23). After incubating *Lactobacillus leichmannii* and *E. coli* for 10–16 hr in Co^{60} vitamin B_{12} enriched media and fractionating the bacteria they found in excess of 90% of the vitamin B_{12} in the ribosomal fraction.

Similar kinetics of vitamin B_{12} uptake by various bacterial species implies similar mechanisms of B_{12} uptake. Grasbeck (8) however, suggested that the pH differences in the B_{12} uptake between *Lactobacillus leichmannii* and *E. coli* reflected differences in the mechanism of uptake.

1 hr B_{12} uptake values for gram-negative bacteria observed in this study are somewhat higher than those reported by Donaldson et al. (2) but similar to those reported by Sherwood, Goldstein, Haurani, and Wirts

(5). It is interesting that two Gram-positive organisms, *B. subtilis* and Group A streptococcus, demonstrated 1 hr B_{12} uptake values (21,700 ±2200 and 1810 ±258 ng respectively) far in excess of those observed for Gram-negative bacteria (6.6 ±1.0 – 255 ±35.9 ng). Both *B. subtilis* and Group A streptococcus were also capable of measurable vitamin B_{12} uptake with as few as 6×10^5 and 5×10^4 organisms respectively, 2000–10,000 times fewer than the number required for the Gram-negative enteric organisms. Thus, it is apparent that organisms other than the so-called "enteric organisms" were capable of substantial vitamin B_{12} uptake. The inability of *Strep. fecalis* to take up vitamin B_{12} in 1 hr is unexplained and paradoxical in view of the fact that it shares many characteristics in common with other streptococci demonstrating B_{12} uptake. Perhaps its vitamin B_{12} uptake is merely much slower than that of the other organisms studied, or alternatively, it may not possess surface receptor sites capable of immediate vitamin B_{12} uptake. Similar behavior of *Strep. fecalis* has been observed by others (2, 3, 5, 24).

The wide range in both first and second stage B_{12} uptake values among the various organisms studied is unexplained. It does not appear to be related to either a requirement for exogenous B_{12} or to ability to synthesize B_{12}. All the organisms examined have been shown not to require exogenous B_{12} (18, 25–28). This study, as well as that of others (12, 18, 25), confirms the fact that a wide variety of bacteria, not requiring exogenous B_{12} for growth, take up as much B_{12} as quickly and by a similar mechanism, as B_{12} requiring organisms. Differing bacterial avidities and capacities for B_{12} must remain unexplained at present.

Avidities of bacteria for B_{12} comparable to that of intrinsic factor for B_{12}, implies that bacteria resident in "blind-loops" may be able to compete with intrinsic factor for ingested B_{12}. Such a conclusion is supported by Donaldson's studies of rats with blind-loops (1, 2). Furthermore, while a variety of bacteria are capable of B_{12} uptake, the differences noted in both avidity and capacity for B_{12} uptake, suggest that overgrowth with certain organisms may more frequently lead to vitamin B_{12} malabsorption. While anaerobes have been shown to be a prominent part of the flora of blind-loops (29–32) their avidity and capacity for B_{12} uptake and their role on the B_{12} malabsorption remains to be elucidated. Thus, in patients with intestinal bacterial overgrowth, a variety of organisms may compete with one another and the host mucosa for available vitamin B_{12}.

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Serum Vitamin B_{12} Content and Unsaturated Vitamin B_{12}-Binding Capacity in Myeloproliferative Disease

Value in Differential Diagnosis and as Indicators of Disease Activity

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SUMMARY The serum unsaturated vitamin B_{12}-binding capacity (UBBC) was found to be of value in distinguishing polycythemia vera from other conditions that are accompanied by an elevated hematocrit or an absolute erythrocytosis and as an indicator of disease activity in polycythemia vera. The serum vitamin B_{12} level was of lesser value. Increased levels of serum vitamin B_{12} were observed in 36% and increased levels of UBBC in 70% of patients with untreated polycythemia vera and related myeloproliferative disorders, as contrasted with normal serum vitamin B_{12} levels in 98% and normal UBBC in 87% of cases of spurious (relative) polycythemia and isolated erythrocytosis (secondary and unclassified polycythemia). Serum vitamin B_{12} and UBBC levels in polycythemia vera treated only with phlebotomy did not differ from values in untreated cases, whereas patients whose erythroid activity had been controlled by myelosuppressive therapy had levels comparable to those in normal subjects. UBBC values were positively correlated with erythroid activity and leukocyte count. Serial observations indicated that changes in UBBC accompanied and in some cases preceded the occurrence of response and relapse in patients receiving myelosuppressive therapy. Changes in serum B_{12} levels did not parallel the erythroid response to therapy, nor were B_{12} levels correlated with the blood count, spleen size, or uric acid values. Chromatographic separation of unsaturated B_{12} binders showed an increase in both alpha and beta components. The frequent observation of elevated B_{12} binders without a concomitant rise in serum B_{12} content in patients with polycythemia vera suggests that some of the B_{12}-binding protein of alpha mobility that is elaborated is abnormal in that it does not bind endogenous vitmain B_{12}.

AN ENHANCEMENT of the vitamin B_{12} levels and binding capacity of plasma and serum due to the presence of increased amounts of B_{12}-binding protein has been described in chronic myelogenous leukemia as well as in other myeloproliferative disorders (1–3). The development of a rapid charcoal assay, which has simplified the determination of serum B_{12} and unsaturated B_{12}-binding capacity (UBBC), prompted a reexamination of levels in patients with polycythemia vera, related myeloproliferative disorders, and those conditions that must be differentiated from polycythemia vera. Preliminary results confirmed that elevations in serum B_{12} and UBBC were frequent in patients with myeloproliferative disorders (4). This paper reports the results of studies in which the specificity of these elevations and their relation to other manifestations of the panmyelosis of myeloproliferative disease were assessed in order to determine the value of

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serum B_{12} and UBBC measurements as indicators in the differential diagnosis of polycythemic states and as indicators of disease activity during the treatment of polycythemia vera.

Patients and Methods

Studies were of patients seen in the Polycythemia Clinic of The Mount Sinai Hospital. In addition to a history and physical examination the following laboratory determinations were performed: [1] measurement of the red cell mass by direct red cell tagging with ^{51}Cr (normal for men, <36 ml/kg of body weight and for women, <32 ml/kg body weight); [2] hematocrit, hemoglobin, red cell count, reticulocyte count, white cell count, white cell differential, and platelet count (phase contrast method —values >400,000/mm³ were considered as elevated); [3] leukocyte alkaline phosphatase score (Kaplow method (5))—a score above 100 in the absence of fever or infection was considered to be elevated; [4] serum uric acid; [5] serum iron and unsaturated iron-binding capacity (6, 7); [6] serum vitamin B_{12} level using the hemoglobin-coated charcoal method of Lau and colleagues (8, 9) and UBBC of serum by the albumin-coated charcoal method of Gottlieb, Lau, Wasserman, and Herbert (10). In certain cases the percentage of B_{12}-binding protein due to alpha and beta globulin was determined by the method, of Retief, Gottlieb, Kochwa, and Pratt (11) and Silverstein and Herbert (12).

The 166 patients studied were assigned to diagnostic categories according to the following criteria:

1. Normal: hematocrit 52% or less; normal red cell mass in the absence of recent blood loss or evidence of iron deficiency; no splenomegaly; normal white cell count, platelet count, and leukocyte alkaline phosphatase score.
2. Spurious polycythemia: hematocrit above 52% with a normal red cell mass in the absence of recent blood loss or evidence of iron deficiency; no splenomegaly; normal white cell count, platelet count, and leukocyte alkaline phosphatase score.
3. Polycythemia vera: increased red cell mass and splenomegaly or, in the absence of splenomegaly, the presence of two or more corroborative abnormalities (elevated platelet count, white cell count, or leukocyte alkaline phosphatase score).
4. Secondary erythrocytosis: increased red cell mass in the presence of diminished arterial oxygen saturation or any condition that may be accompanied by increased erythropoietin elaboration; absence of splenomegaly, thrombocytosis, leukocytosis, or elevated leukocyte alkaline phosphatase score.
5. Unclassified erythrocytosis: isolated increase in red cell mass without leukocytosis, thrombocytosis, elevated leukocyte alkaline phosphatase score, or splenomegaly; normal arterial oxygen saturation; absence of any demonstrable lesion known to be associated with secondary erythrocytosis.
6. Spent polycythemia vera: a history of documented polycythemia vera followed by a decrease of the red cell mass to normal levels or below in the absence of bleeding or myelosuppressive therapy, and increasing extramedullary hemopoiesis as evidenced by an enlarging spleen or liver, or both, and a leukoerythroblastic blood picture.
7. Myeloid metaplasia, myelofibrosis: no history of polycythemia vera; a low or normal red cell mass in the absence of bleeding or myelosuppressive therapy; splenomegaly or hepatomegaly, or both, with a leukoerythroblastic blood picture and fibrosis of the marrow.

Cases of polycythemia were staged on the basis of their erythroid activity as follows:

1. Polycythemia vera active, untreated: patients with increased erythroid activity as evidenced by a hematocrit above 52% who had received no recent phlebotomy or myelosuppressive therapy. This group comprised newly diagnosed patients and those in relapse after prior myelosuppressive therapy.
2. Polycythemia vera phlebotomized: patients with increased erythroid activity who had been phlebotomized after the rise in hematocrit.
3. Polycythemia vera treated without erythroid response: patients receiving myelosuppressive therapy without a response in the hematocrit. (Agents used were chlorambucil cyclophosphamide, busulfan, ^{32}P). Since the effect of myelosuppressive therapy on granulopoiesis and megakaryocytic proliferation may occur in the absence of a change in hematocrit these patients showed various degrees of response to their leukocyte and platelet counts despite the lack of erythroid response.
4. Polycythemia vera controlled: patients in whom the hematocrit had stabilized at 52% or below without phlebotomy in response to myelosuppressive therapy.

Results

The B_{12} and UBBC levels in the 166 patients studied are summarized in Table 1

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TABLE 1. Serum B_{12} and Unsaturated B_{12}-Binding Capacity (UBBC) in Normal Subjects and Patients with Spurious Polycythemia, Erythrocytosis, and Various Stages of Myeloproliferative Disease

Diagnosis	Number of Patients	Mean Age (Range)	Serum B_{12} (Mean ± SD)	Serum UBBC (Mean ± SD)
		yr	*pg/ml*	
Normal	28	51 (27–70)	445 ± 245	1,605 ± 386
Spurious polycythemia	32	58 (41–81)	391 ± 217	1,662 ± 438
Secondary erythrocytosis	8	59 (35–73)	545 ± 171	2,040 ± 465
Unclassified erythrocytosis	7	47 (22–74)	435 ± 171	1,583 ± 354
Polycythemia vera				
I active, untreated	22	61 (46–78)	984 ± 1,072	3,215 ± 1,462
II active, phlebotomized	16	61 (32–78)	1,009 ± 865	3,012 ± 1,219
III active, on myelosuppressive therapy, no erythroid response	7	61 (50–71)	517 ± 335	2,863 ± 883
IV controlled	27	57 (27–73)	494 ± 325	1,803 ± 594
Spent polycythemia vera	10	63 (48–73)	977 ± 380	3,164 ± 1,038
Myeloid metaplasia, myelofibrosis	9	66 (44–80)	1,082 ± 623	3,112 ± 1,343

and Figures 1 and 2. For this analysis each patient was included only once and was classified according to his status at the time when the first B_{12} and UBBC determinations were performed. Comparable means were obtained using the status and values from the last visit or the average of serial tests performed while the patient's status was unchanged. Elevated levels of vitamin B_{12} and UBBC were found in patients with active polycythemia vera (whether untreated or treated with phlebotomy), spent polycythemia vera, myeloid metaplasia, and myelofibrosis as contrasted with normal values seen in patients with spurious polycythemia and isolated erythrocytosis. Patients in whom myelosuppressive therapy had produced control of erythroid activity had levels comparable to those seen in normal subjects. Patients who were receiving myelosuppressive therapy but who showed no response as regards control of erythroid activity had normal serum B_{12} levels accompanied by elevated UBBC values.

Table 2 shows the incidence of elevations in serum vitamin B_{12} and UBBC to levels greater than 2 SD above the normal mean in polycythemic states unrelated to myeloproliferative disease (spurious or relative polycythemia, secondary and unclassified erythrocytosis) and in myeloproliferative states (at a time when the panmyelosis was not being controlled by myelosuppressive therapy). A significant number of patients with myeloproliferative disease had elevated levels of vitamin B_{12} (36%, $P < 0.01$) and of UBBC (70%, $P < 0.001$) in contrast to normal subjects, patients having elevated hematocrit levels without evidence of myeloproliferative disease, and patients

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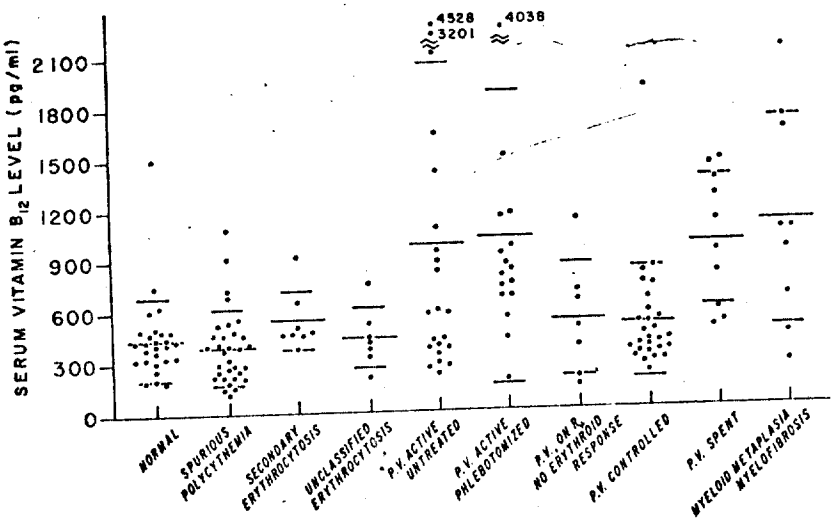

FIGURE 1. Serum vitamin B_{12} levels in a population of normal subjects, patients with hematocrit elevations not due to myeloproliferative disease, and patients with various stages of myeloproliferative disease. The mean levels ±1 SD are indicated by horizontal lines. In the "P.V. (polycythemia vera) active, untreated" group the mean −1 SD was less than zero and is not plotted.

with polycythemia vera in whom erythroid proliferation had been successfully controlled with myelosuppressive therapy.

The effect of treatment was studied further by comparing the B_{12} and UBBC levels in a sample of 71 patients, 38 of whom

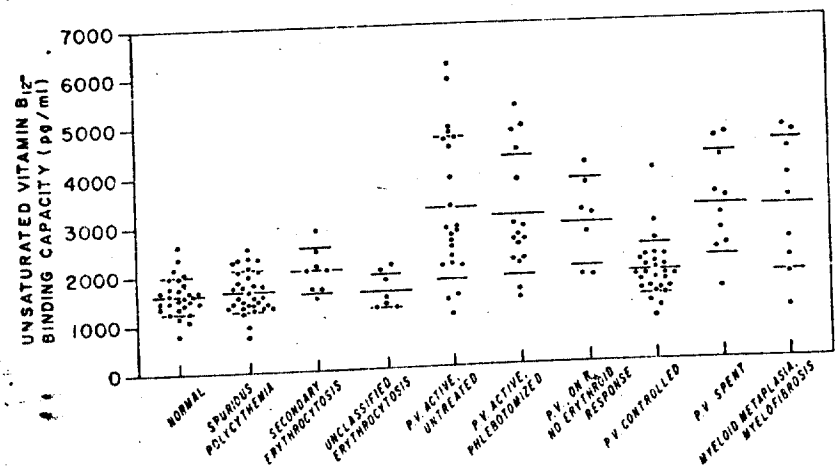

FIGURE 2. Serum levels of unsaturated vitamin B_{12}-binding capacity in a population of normal subjects, patients with hematocrit elevations not due to myeloproliferative disease, and patients with various stages of myeloproliferative disease. The mean levels ±1 SD are indicated by horizontal lines. P.V. = polycythemia vera.

TABLE 2. Incidence of Elevated Serum Vitamin B_{12} Levels and Serum Unsaturated Vitamin B_{12}-Binding Capacity (UBBC) in Normal Subjects, Patients with Hematocrit Elevations Not Due to Myeloproliferative Disease, and Patients with Various Stages of Myeloproliferative Disease

Diagnostic Group	Serum Vitamin B_{12}		UBBC	
	Number Elevated* / Number Studied	Percent Elevated†	Number Elevated* / Number Studied	Percent Elevated†
Normal	2/28	7	1/28	4
Elevated hematocrit, no myeloproliferative disease: (spurious polycythemia, secondary erythrocytosis, unclassified erythrocytosis)	1/47	2	6/47	13
Myeloproliferative disease: (polycythemia vera active, untreated and phlebotomized; polycythemia vera on treatment, no erythroid response; polycythemia vera spent; myeloid metaplasia, myelofibrosis)	23/64	36‡	45/64	70§
Polycythemia vera, controlled with myelosuppressive therapy	1/27	4	3/27	11

* Values greater than the mean + 2 SD of the normal group.
† Level of significance of comparison with normal subjects (based on chi-square value).
‡ $P < 0.01$.
§ $P < 0.001$.

were seen during only 1 stage of their disease and 33 of whom were studied serially during 2 or more stages. Only 1 determination from each patient during each stage was included, giving a total of 125 stages that were analyzed. The data presented are the means of the first visit of each stage. Comparable values were obtained using the last visit and the average of serial visits made while the status was unchanged*.

The Effect of Phlebotomy: No significant differences in the level of B_{12} or UBBC were observed when untreated patients with active polycythemia vera were compared with patients who had been phlebotomized (Table 3).

The Effect of Myelosuppressive Therapy (Table 4): Both B_{12} and UBBC levels were significantly lower during control with myelosuppressive therapy than during the active, untreated stage. B_{12} levels tended to be lower and not significantly different from normal in patients who were receiving or had received myelosuppressive therapy than in previously untreated subjects whether or not erythroid activity had responded to therapy. UBBC levels, however, were elevated in patients with active disease, whether previously untreated or dur-

* It is appreciated that the inclusion of repeated observation in single individuals introduces some biases into the study, in that a controlled patient whose status is unchanged would contribute fewer stages to the series than an active patient whose disease is undergoing dynamic changes and who may contribute three or more serial stages. In view of the relatively limited population with polycythemia vera that is available and the prolonged course of the disease, this objection may be balanced by the advantages of using all the data obtained in a relatively small population to yield a kaleidoscopic picture of changes that occur during the various stages of the disease.

TABLE 3. The Effect of Phlebotomy on Serum B_{12} and Unsaturated B_{12}-Binding Capacity (UBBC)

Status	Number of Stages Observed	Serum B_{12} (Mean ± SD)	Serum UBBC (Mean ± SD)
		pg/ml	
Untreated	34	865 ± 778	3,235 ± 1,395
Phlebotomized	25	875 ± 841	3,065 ± 1,293

TABLE 4. The Effect of Myelosuppressive Therapy on Serum B_{12} and Unsaturated B_{12}-Binding Capacity (UBBC) in 71 Patients Observed During 125 Stages of Polycythemia Vera

Status	Number of Stages Observed	Serum B_{12} (Mean ± SD*)	Serum UBBC (Mean ± SD*)
		pg/ml	
Active, no previous myelosuppressive therapy	26	1,068 ± 1,089	3,034 ± 1,244
Active, in relapse, previous myelosuppressive therapy	33	715 ± 363†	3,301 ± 1,422
Receiving myelosuppressive therapy without a response in hematocrit	20	725 ± 445†	3,027 ± 861
Controlled	46	619 ± 501‡	2,108 ± 818§

* P value of comparison with levels in active patients with no previous myelosuppressive therapy (one-tailed t test).
† $P < 0.05$.
‡ $P < 0.01$.
§ $P < 0.001$.

ing relapse after remission induced by myelosuppressive therapy, as well as during the administration of myelosuppressive therapy if a response in hematocrit had not occurred. Only in the controlled group were mean levels of UBBC comparable to those in normal subjects. Myelosuppressive therapy per se was not responsible for the lower values, since in patients being treated by a regimen of intermittent chemotherapy a comparison of measurements taken while the patient was on and off drugs showed no significant differences in B_{12} or UBBC levels in the absence of a change in status. Serial studies of individual patients corroborated these observations as shown in Figure 3.

In the previous analysis the staging of patients with polycythemia vera was based on the degree of erythrocytic proliferation present. Since several hematic cell populations are involved in the panmyelosis of polycythemia vera and since these may also be affected by myelosuppressive therapy, the relative influence of the various types of proliferative activity that may occur simultaneously in polycythemia vera on B_{12} and UBBC levels was studied. A summary chi-square analysis (Mantel and Haenszel (13)) was performed using the data from patients studied during various stages of their disease. Since it is advantageous to avoid the inclusion of multiple variables that are strongly interrelated (for example, hemoglobin, red cell count, and hematocrit) in such an analysis, a new variable "erythroid activity," was substituted. Erythroid activity was considered as "normal" if the hematocrit was 52% or below in the absence of recent phlebotomy or bleeding and "increased" in the presence of a hematocrit above 52% or if phlebotomy was performed for an elevated hematocrit during the previous 2 months regardless of the hematocrit level at the time of sampling. The other variables included in the analysis were also dichotomized and classified as "normal" or "increased." B_{12} and UBBC levels were then examined separately with respect to the influence of each of the following: erythroid activity, white cell count, platelet count, serum uric acid level, and size of spleen. With each analysis an adjustment was made for simultaneous changes in the variables other than the ones under study. (The association between two variables such as B_{12} and white cell count was evaluated by subclassification of the data

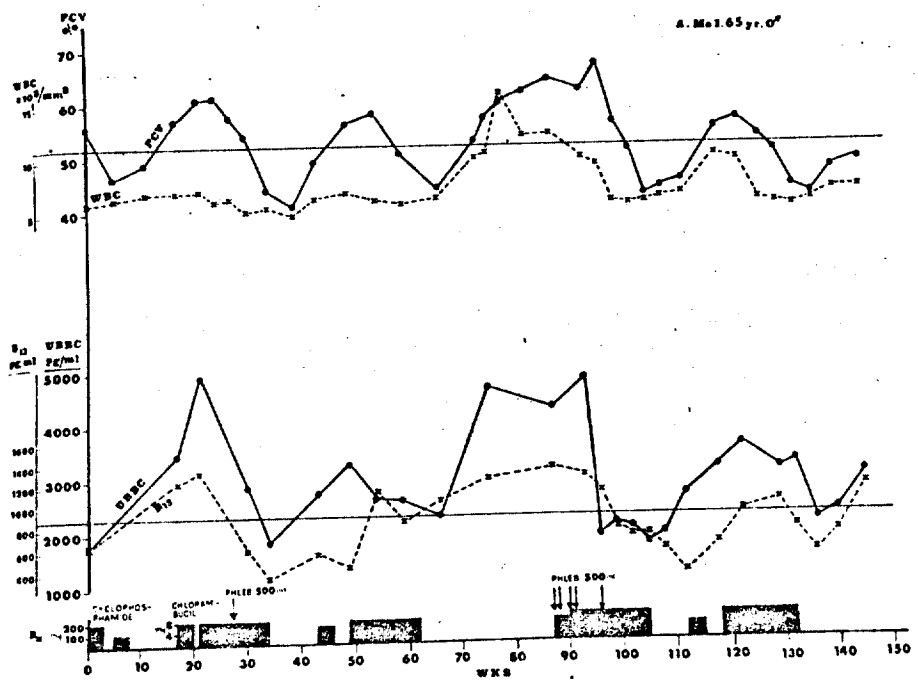

FIGURE 3. The effect of myelosuppressive therapy on levels of serum vitamin B_{12} and unsaturated B_{12}-binding capacity (*UBBC*) in polycythemia vera. The patient was observed during five courses of therapy (one with cyclophosphamide, four with chlorambucil). B_{12} and UBBC levels rose during relapse after cessation of drug therapy and fell when treatment was reinstituted. Note that during three of the four relapses a rise in UBBC occurred in the pesence of a normal peripheral white cell count. PHLEB = phlebotomy; PCV = packed cell volume or hematocrit.

that is, holding the other variables constant —so that a one degree of freedom chi-square statistic could be calculated based on the comparison of the summation of observed and expected values over all subclasses.) After such adjustments the analyses showed no statistically significant association between the B_{12} values and any of the variables analyzed. In contrast, UBBC levels were significantly correlated with erythroid activity and white cell count (Table 5).

In view of this correlation, we sought to establish whether the UBBC determination provided any additional information regarding the status of the patient beyond that obtained from measurements of the hematocrit and the white count. Table 6 summarizes the frequency with which an elevated UBBC (> 2,377 pg/ml, the mean

TABLE 5. The Relation of B_{12} and Unsaturated B_{12}-Binding Capacity (UBBC) Levels to the Manifestations of Myeloproliferative Disease Activity: Mantel-Haenszel Summary Chi-Square Analysis

	Chi-Square Values	
	B_{12} Level	UBBC Level
Erythroid activity	0.223	10.338*
Platelet count	0.004	2.280
White cell count	0.939	10.205*
Spleen size	0.155	0.659
Serum uric acid	0.181	0.013

* $P < 0.01$.

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TABLE 6. Frequency of Elevated Unsaturated B_{12}-Binding Capacity (UBBC) Level in Association with Normal White Count in the Course of Myeloproliferative Disease

Stage	Number of Stages Observed	Stages in Which UBBC >2,377 pg/ml and WBC* <12,000/mm³
		no. (%)
Polycythemia vera active	59	16 (27%)
Polycythemia vera treated, no response	20	9 (45%)
Polycythemia vera controlled	46	13 (28%)
Spent polycythemia vera	10	5 (50%)
Myeloid metaplasia, myelofibrosis	9	2 (22%)

* White blood cell count.

plus 2 SD in normal subjects) occurred in association with a white count below 12,000/mm³. This association was observed in 34 of 90 patients with myeloproliferative disease (38%) at some time during their course and occurred with the greatest frequency in patients with spent polycythemia vera and in those who were receiving myelosuppressive therapy without a response in hematocrit level.

The alpha and beta components of the unsaturated B_{12}-binding protein of serum were separated by diethylaminoethanol cellulose chromatography and quantitated in 33 patients with myeloproliferative disease (Table 7). Increased amounts of alpha globulin binder (> 301 pg/ml) were recovered in 91% and increased amounts of beta globulin (> 1,093 pg/ml), in 71% of the patients studied. A relatively greater increase in alpha globulin binder was observed, these binders constituting an average of 31.7 ± 12.5% of the total unsaturated binding protein as compared with normal subjects in whom 18.8% of the UBBC was characterized as alpha globulin. Although modest increases in the absolute amount and relative percentage of alpha binder were observed, these were by no means of the same magnitude as occurs in chronic myelocytic leukemia (*see* Table 7), whereas the elevation of beta globulin levels were comparable in polycythemia vera and chronic myeloid leukemia.

DISCUSSION

The results indicate that the serum B_{12} content and the capacity of the serum to

TABLE 7. Distribution of Human Serum Unsaturated B_{12}-Binding Capacity (UBBC) Between Alpha and Beta Globulin Fractions of "Baby Column" Chromatography

Diagnosis	Number of Subjects	Serum B_{12} Level Mean (Range)	Unsaturated Alpha Globulin Mean (Range)	Unsaturated Beta Globulin Mean (Range)	Percent Alpha	Percent Beta
		←	*pg/ml*	→		
Normal	5	387 (258–584)	216 (119–301)	933 (731–1,093)	18.8 (12.1–29.1)	81.2 (70.9–87.9)
Polycythemia vera active	9	1,341 (376–4,528)	1,142 (412–2,557)	2,337 (1,114–3,675)	31.8 (21.1–54.4)	68.2 (45.6–78.9)
Polycythemia vera controlled	6	625 (418–1,339)	697 (298–1,570)	1,362 (634–2,494)	32.0 (22.9–45.3)	68.0 (54.7–77.1)
Polycythemia vera spent	10	1,739 (633–4,038)	813 (440–1,530)	1,949 (731–3,708)	31.9 (15.1–60.2)	68.1 (39.8–84.9)
Myeloid metaplasia	8	861 (247–1,727)	587 (243–1,490)	1,481 (370–4,025)	31.0 (12.9–58.8)	69.0 (41.2–87.1)
Chronic myeloid leukemia	5	4,484 (2,097–8,810)	10,902 (6,760–17,170)	2,346 (745–5,964)	82.9 (68.6–97.3)	17.1 (2.7–31.1)

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bind additional vitamin B_{12}, added in vitro (expressed as UBBC), are increased in polycythemia vera and related myeloproliferative states. Such abnormalities are not features of those diseases in which isolated erythrocytosis occurs, nor are they present in subjects with an elevated hematocrit and a normal red cell volume (spurious or relative polycythemia). The significant increase in the incidence of UBBC elevations (70%) in patients with myeloproliferative disease who were not receiving myelosuppressive therapy over that in a normal population and a group of patients with polycythemic states not related to myeloproliferative disease suggests that the UBBC is a useful indicator in distinguishing polycythemia vera from other conditions that are accompanied by an elevated hematocrit or an absolute erythrocytosis, or both. The serum vitamin B_{12} level, although elevated in 36% of patients with myeloproliferative disease, would appear to be of lesser value in differential diagnosis.

The study has also shown that in patients with polycythemia vera the serum B_{12} and UBBC levels are related to the status of the disease. In patients with active disease who had received no therapy both B_{12} and UBBC were significantly elevated. The serum B_{12} was lower in subjects treated with myelosuppressive therapy (regardless of the response in hematocrit levels) than in untreated patients or those being treated only with phlebotomy. In patients during relapse after cessation of myelosuppressive therapy levels of B_{12} were also significantly lower than in patients with active disease who were previously untreated. Even more striking effects on UBBC levels were produced by myelosuppressive therapy, and the UBBC appears to reflect changes in the patient's status in response to treatment more accurately than does the B_{12} level. The UBBC was elevated both in patients with active disease (even in relapse after cessation of myelosuppressive therapy) and in patients receiving myelosuppressive therapy without successful control of their erythroid activity. In contrast, significantly lower levels of UBBC were observed ($P < 0.001$) during the controlled stage. In some patients studied serially UBBC levels were found to be a sensitive indicator of impending relapse, rising before changes in the blood count or spleen size occurred.

Although a strongly positive association was observed between UBBC and erythroid activity and UBBC and leukocyte count, the UBBC was elevated in the presence of a normal leukocyte count in 38% of patients with polycythemia vera at some time during their course. Thus, it appears that the UBBC level may provide additional information regarding the status of disease beyond what can be gained from the determination of the hematocrit and white cell count.

The source of the increased amounts of B_{12} binder that appear in the serum of patients with myeloproliferative disease is not known. Chromatographic separation has shown that the levels of both alpha and beta globulin binders are increased and that relatively greater amounts of alpha globulin are present so that the normal ratio of unbound alpha to beta (1:5) is increased to 1:3. B_{12}-binding protein has been isolated from leukocytes (predominantly granulocytes) and has a molecular weight and immunologic characteristics similar to those of alpha globulin B_{12} binder of serum (14, 15). Simons and Weber (15) have demonstrated that granulocytes are capable of synthesizing this protein.

As part of the panmyelosis of polycythemia vera and myeloid metaplasia, increased numbers of granulocytes are produced and destroyed. An accumulation of B_{12}-binding protein in the serum might be anticipated as a result of this increased granulocyte turnover. This hypothesis is compatible with the return to normal of the UBBC during control of polycythemia vera with myelosuppressive therapy and the good

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correlation observed between UBBC levels and the leukocyte count. The same mechanism has been invoked to explain the marked increase in the B_{12}-binding capacity of chronic myelocytic leukemia serum (16). In chronic myeloid leukemia the elevated binding capacity of serum is attributable primarily to an increase in alpha globulin that may comprise as much as 97% of the UBBC. The amount of beta binder present is normal or only modestly elevated. The affinity for endogenous B_{12}, characteristic of normal alpha binder, appears to be retained in myeloid leukemia, and as the UBBC rises a concomitant increase in serum B_{12} content is observed. In contrast, a dissociation between B_{12} and UBBC levels was common in polycythemia and myeloid metaplasia.

In 70% of the instances in which the UBBC was greater than 2,377 pg/ml (2 SD above the normal mean) the serum B_{12} was within the normal range (< 935 pg/ml). An hypothesis that might explain such a dissociation is that the increased B_{12}-binding capacity is due to the presence of an abnormal binder that migrates with the alpha fraction in the system used for globulin separation in this study but that differs from normal alpha binder in having a low affinity for endogenous B_{12}. This protein would resemble beta binder, which is free from B_{12} in vivo. Hall and Finkler (17) reported that the elevated B_{12} binder in polycythemia vera serum described by Gottlieb, Retief, Pratt, and Herbert (16), which does not carry endogenous B_{12}, is actually two binders. In his system of separation both have the electrophoretic mobility at pH 8.6 of beta globulin. One of these two binders migrates distinct from alpha and beta binders at pH 4.5 and has a higher molecular weight than the beta binder found in normal serum. The description of such a protein supports the concept that the presence of an abnormal B_{12} binder may account for the observed discrepancy of normal B_{12} levels in the presence of elevated UBBC levels.

The increase in both alpha and beta binders in polycythemia vera remains to be explained, since granulocytes have been thought to elaborate primarily alpha globulin. Stenman, Simons, and Gräsbeck (18) have reported recently that purification or treatment with neuramidase changes the electrophoretic properties of B_{12}-binding protein derived from myeloid leukemia serum (originally $alpha_2$ mobility) to beta mobility. They suggest that the differences in mobility between the serum and leukocyte binder in chronic leukemia may be due to the sialic acid content and that sialic acid might be added to the protein at the stage of secretion from the leukocytes into the plasma, thus accounting for the change in mobility. The demonstration of such a relationship between alpha and beta binders suggests that one mechanism for the production of abnormalities in UBBC in myeloproliferative disease may be a qualitative defect in some of the leukocytes that results in the elaboration of B_{12} binders with an altered mobility and an impaired ability to bind endogenous B_{12}.

Acknowledgments

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Epidermal Changes in Vitamin B_{12} Deficiency

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A young white girl had a peculiar poikilodermatous pigmentary disturbance and a megaloblastic anemia. Both changes were resolved with vitamin B_{12} therapy. Epidermal cells in areas of pigmentation were found to have abnormally large nuclei similar to those previously described in the mucosal epithelium of patients with vitamin B_{12} deficiency. The epidermal nuclear size returned to normal following treatment, supporting the assumption that these enlarged nuclei were indicative of the deficiency. The data suggest that a common mechanism linked with the vitamin B_{12} deficiency state may be responsible for both the pigmentary disturbance and the cytologic abnormality. The absence of cytologic changes in the skin of normally pigmented areas in this patient and in a second case of vitamin B_{12} deficiency without a pigmentary disturbance is consistent with this theory.

Reversible cutaneous pigmentation is recognized as an occasional event in vitamin B_{12} deficiency.[1-3] In 1963, Baker et al[3] described hyperpigmentation as a sign of vitamin B_{12} deficiency and noted its frequent occurrence in the dark-skinned races. Indeed, almost all reports of this association have involved natives of Africa[4,5] or India.[3,6] There is but a single case report in recent times describing the occurrence of pigmentation in a vitamin B_{12}-deficient white child.[7]

The importance of the association between hyperpigmentation and vitamin B_{12} deficiency rests in its reversibility with vitamin B_{12} replacement. The mechanism for this reversible pigmentation is unclear. Baker et al[8] were unable to show elevation of melanocyte-stimulating hormone levels, and abnormalities of adrenal function have not been found.[9] Little other biochemical data are available. In addition, morphologic changes in the cells of the epidermis have not been described, although epithelial cells from various mucosal surfaces show characteristic changes in megaloblastic anemia.[10-22] The present study was stimulated by our examination of a patient with vitamin B_{12} deficiency and skin pigmentation. The pathologic findings led to a quantitative analysis of epidermal nuclear size prior to and following therapy.

Report of a Case

An 8-year-old white girl was seen in 1966 with a chief complaint of discoloration of the skin, which had begun at age 8 months as symmetrical hyperpigmentation of the dorsal aspect of her fingers. Progressive involvement subsequently occurred which initially appeared related to sun exposure. Her growth, development, and general health were good. In 1964, she was taken to a physician for evaluation of the pigmentary disorder. Except for the presence of mottled pigmentation of the neck, axilla, groin, and lateral abdomen, the results of a physical examination were unremarkable. Extensive studies failed to identify a cause for the increased pigment. The only laboratory abnormality was a hemoglobin value of 11.5 gm/100 ml. Over the next two years, the pigmentation gradually increased, she began to have recurrent respiratory infections, and she became progressively more anemic. This led to referral to the Stanford Medical Center in 1966.

On physical examination, she was listless, pale, and thin. The only distinctive finding was the cutaneous pigmentation (Fig 1).

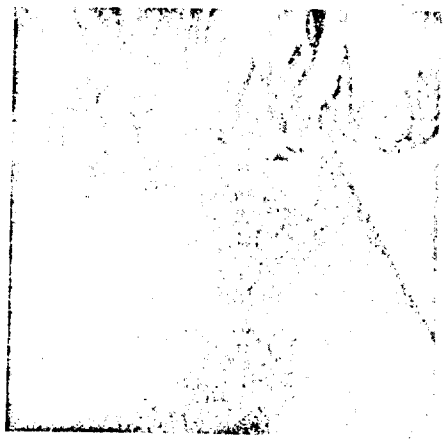
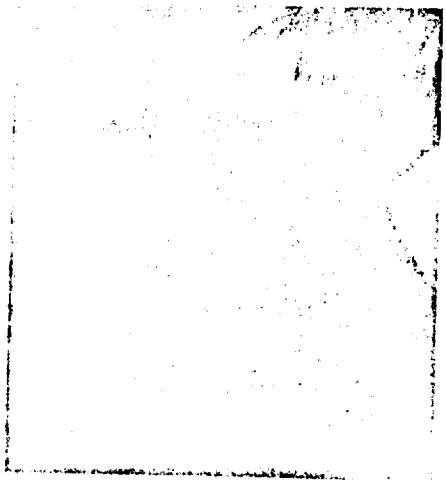


Fig 1.—Skin before vitamin B_{12} replacement. Widespread mottled pigmentation simulated that in arsenical dermatosis. This change was most severe over neck, lateral abdomen, groins axillae, and flexor surface of the extremities.

Fig 2.—Striking resolution of pigmentary disturbance 14 months after vitamin B_{12} replacement.



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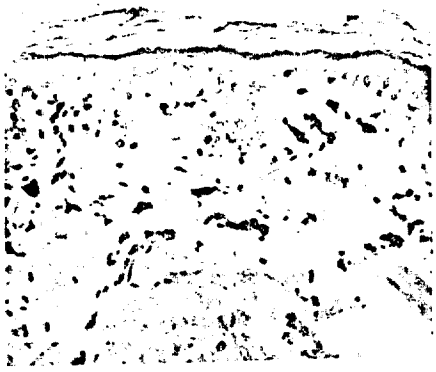

Fig 3.—Hyperpigmentation before treatment, showing epidermal atrophy, dystrophy, and marked pigmentary incontinence (hematoxylin-eosin, ×270).

Fig 4.—Ferric ferricyanide technique demonstrating the distribution of reducing granules in epidermal melanocytes and dermal melanophores (×400).

Table 1.—Laboratory Data*

	1964	Hospital Day† 1966				1970
		1st	4th	10th	20th	
Hgb, gm/100 ml	11.5	5.9	4.3	8.1	10.5	14.1
Hct, vol%	...	...	...	...	...	41
Blood smear		Diminished platelets, hypersegmented polymorphonuclear leukocytes and macrocytosis				Normal
WBC count, cu mm	...	2,700	...	...	6,300	6,500
Reticulocytes, %	...	0.1	...	18	...	...
Platelets, cu mm	...	72,000	...	...	256,000	Normal
Total serum bilirubin, mg/100 ml	...	1.2	...	...	...	...
Total serum iron, μg/100 ml	...	221	...	...	60	86
Total iron-binding capacity, μg/100 ml	...	276	...	...	...	366
Serum folate, mμg	...	2.4 (normal, 3-27)	...	...	...	...
Serum vitamin B_{12}, μμg	...	80 (normal, 150-1,500)	...	...	...	...
Schilling's test	...	4% excretion/24 hr, 4% excretion/24 hr with intrinsic factor (single and double dose)	...	...	...	4%/24 hr, 4%/24 with intrinsic factor
Gastric acid	...	Present	...	...	...	...
Gastric biopsy	...	Normal	...	...	...	...
Bone marrow	...	Frank megaloblastic dyspoiesis	...	...	...	...
Intrinsic factor Ab	...	Negative	...	...	...	Negative

* Normal values were obtained for the following: electrolytes; blood urea nitrogen; calcium; phosphate; alkaline phosphatase (King-Armstrong); serum glutamic oxaloacetic transaminase, urinary steroids; serum proteins; urinary amino acid screen; protein-bound iodine; glucose; uric acid; cholesterol; glucose-6-phosphate dehydrogenase; arsenic levels (hair and nails); 72-hour stool fat; D-xylose test; serum carotine; and urinalysis.

† Vitamin B_{12} treatment extended from the fourth hospital day, 1966, through 1970.

Hospital Evaluation and Course.—A summary of the laboratory findings is listed in Table 1. A diagnosis of vitamin B_{12} deficiency was established by the classic findings of the peripheral smear and marrow in addition to a low serum vitamin B_{12} level. The pathophysiologic mechanisms for this deficiency were explored. The patient was found to have free gastric acid, normal intrinsic factor activity, and normal gastric mucosal structure at the time of biopsy. Gastrointestinal x-ray film studies failed to demonstrate any abnormality. Results of a 72-hour stool fat analysis, D-xylose absorption, and serum carotene levels were normal. Intrinsic factor antibodies could not be demonstrated. It was concluded that the mechanism of the vitamin B_{12} deficiency was an isolated defect in vitamin B_{12} absorption.

The anemia responded dramatically to the parenteral administration of 1,000μg of cyanocobalamin. The reticulocyte count promptly rose to a peak of 18% five days later. Over the first two weeks of therapy, the hemoglobin level rose from 4.3 to 10.5 gm/100 ml. The thrombocytopenia and leukopenia disappeared, and there was remarkable resolution of the abnormal pigmentation during the subsequent year (Fig 2).

In April 1970, the patient was readmitted for followup evaluation. She had been receiving monthly cyanocobalamin injections since her admission four years before, and had had no significant illness. The anemia and pathological pigmentation had completely disappeared.

Special Studies

Tissues.—Skin biopsy specimens were obtained from areas of hyperpigmentation and from normal skin prior to and following vitamin B_{12} replacement. Posttreatment biopsies were done when all hematologic factors were normal. In addition to hematoxylin-eosin, the following staining and histochemical procedures were applied to sections of tissue that were fixed in formaldehyde solution and embedded in paraffin: Verhoeff-van Gieson technique, PAS reaction, and Prussian blue reaction for iron and ferric ferricyanide reduction technique for the identification of melanin and other reducing granules.

Nuclear Size Studies.—Images of slides stained with hematoxylin-eosin were projected onto paper at a magnification of 1,200 times. Two hundred malpighian cell nuclei were traced consecutively. Pyknotic or condensed nuclei, nuclei with indistinct nuclear membranes, and nuclei that did not show a smooth, oval-to-round contour were avoided. The nuclei from each specimen were numbered sequentially and, using a table of random numbers, 100 of these were selected. The major and minor diameters were measured at right angles and nuclear areas were calculated in a manner similar to that described by Staats et al.[12] The results were expressed in the form of frequency distribution curves. The maximum, minimum, mean, and standard deviation from the mean

were recorded. Repetition of the procedure on different parts of the same specimen yielded essentially the same results.

For comparison, similar measurements were made on a specimen of clinically normal skin from another patient with untreated pernicious anemia, diagnosed at subsequent autopsy by the presence of megaloblasts in the bone marrow and a characteristic gastric lesion. In addition, skin samples from seven individuals without histologically detectable skin disease were studied. Two specimens at the site of intradermal nevi and one with mild epidermal atrophy of unknown cause were examined in like fashion.

Results

Tissues.—The histologic appearance of the specimens from nonpigmented areas of the patient's skin was normal. Specimens from areas of hyperpigmentation showed irregular epidermal atrophy, absence of basal orientation of epidermal cells, patchy pigmentation of the lower epidermis, and numerous pigment-laden macrophages in the upper dermis (Fig 3). Mitotic figures were rarely seen. The ferric ferricyanide technique revealed numerous fine reducing granules in epidermal melanocytes filling the cytoplasm and extending in many directions within dendritic processes. The surrounding keratinocytes, however, were comparatively pigment-free, most of the pigment being in melanophores within the upper dermis (Fig 4). The dermis presented little evidence of inflammation. Occasional round eosinophilic bodies, some containing pyknotic, laterally displaced nuclear remnants, appeared to be damaged epidermal cells. The stratum corneum was not abnormally dense, and there was no parakeratosis.

Nuclear Size Studies.—The data on nuclear areas are summarized in Table 2. All of the control values were similar. There was little variation of nuclear size in relation to age, sex, or body site. The shaded area of Fig 5 is a composite representation of the normal frequency distribution curves. All curves had a narrow base with a mean nuclear area of $34 \pm 3\mu^2$. The data from the control specimens, the case with megaloblastic anemia without hyperpigmentation, and the specimens from nonpigmented skin of our patient are also similar (Table 2). However, the pretreatment specimens from hyperpigmented skin yielded curves with a broader base, indicating more variability in nuclear size and an overall shift to the right and illustrating the nuclear enlargement. These curves resemble those of previous investigators that depict the changes in nuclear size of mucosal epithelial cells in patients with megaloblastic anemia.[10,13,15] The changes were apparent only in the areas of hyperpigmentation when our patient was vitamin B_{12}-deficient. The frequency distribution curve of nuclear areas three years eight months after beginning therapy is normal (Fig 5).

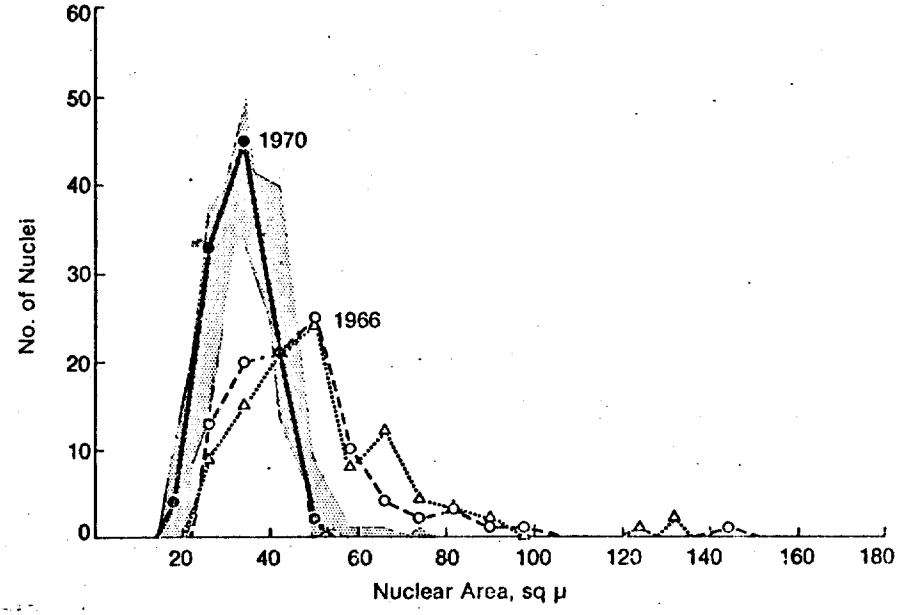

Fig 5.—Distribution curves for nuclear area in normal epidermis (shade zone), the patient's pigmented epidermis in 1966 (circles and triangles), and the posttreatment epidermis in 1970 (solid circles).

Comment

Two noteworthy features of this case are (1) the mechanism of the vitamin B_{12} deficiency, and (2) the character of the skin involvement. Imerslund[23] and Grasbeck et al[24] independently described a syndrome of vitamin B_{12} malabsorption in the presence of normal intrinsic factor activity occurring in infants and young children. The lesion, apparently an ileal defect of vitamin B_{12} absorption, has been assumed to be a congenital anomaly. Most such cases have been diagnosed by age 5 years and many have exhibited proteinuria.[25,26] Fewer than 30 cases have been reported since the original description by Imerslund in 1960. None has had hyperpigmentation. Watson-Williams and Fleming[1] have reported a case with the combination of isolated vitamin B_{12} malabsorption and reversible hyperpigmentation. However, their case, an adult, was not reexamined following therapy to eliminate the possibility of a transient absorptive defect.[27,28] For these reasons, classification of their case is open to question. However, except for the absence of proteinuria, our case does seem to fit into the syndrome described by Imerslund.

Reversible hyperpigmentation has been recognized as a rare complication of both vitamin B_{12} [1-4] and folate deficiency,[5] suggesting some common factor between marrow megaloblastosis and this cutaneous abnormality. Prior to this study, only gross aspects of the skin involvement have been recognized. The pigmentary change has implied some alteration in melanocyte function; however, there have been no data regarding

Table 2.—Data on Nuclear Areas

	Age, yr	Sex	Site of Biopsy	Mean Area, sq μ	SD	Minimum Area, sq μ	Maximum Area, sq μ	Histologic Diagnosis*
Control slides	4	M	Leg	32	8	20	51	IDN
	52	F	Leg	34	7	18	63	NID
	15	F	Heel	34	7	20	74	NID
	7	F	Axilla	35	8	18	56	NID
	32	F	Chest	32	8	14	54	"Normal skin" NID
	18	F	Chest	32	6	17	46	NID
	24	F	Thigh	33	5	25	49	NID
	68	F	Neck	34	6	21	49	Epidermal atrophy
	12	F	Face	33	8	17	59	IDN
	26	M	Chest	37	7	21	57	NID
Mean				34				
Megaloblastic anemia without cutaneous abnormality (PA diagnosed at autopsy	66	M	Leg	35	7	20	59	No abnormal pigment noted
Case studied								
Megaloblastic anemia with pigmentation, nonpigmented (normal) skin, premedication	8	F	Groin	33	7	21	56	Normal appearance
	8	F	Abdomen	33	7	17	60	
Hyperpigmented skin	8	F	Back	47	14	25	98	Marked hyperpigmentation
	8	F	Abdomen	53	14	28	95	
After vitamin B_{12} replacement therapy, 1970, postmedication	12	F	Groin	34	5	25	51	Normal

* IDN, intradermal nevus; NID, no identifiable disease; PA, pernicious anemia.

the effect of the deficiency on the squamous epithelial cell. This study documents the occurrence of cytologic changes in epidermal cells due to vitamin B_{12} deficiency. The findings contribute to the evidence that the metabolic defect induced by this deficiency is body-wide. This generalized disorder of cellular growth has been attributed to an impairment of or delay in DNA synthesis with continued RNA and protein synthesis.[29,30,31] The present findings raise the possibility of a common mechanism leading to both the growth and pigmentary abnormalities. Although a block in DNA synthesis could explain the observed growth changes, it is difficult to account for the pigmentary abnormality on this basis. Therefore, metabolic activities of vitamin B_{12}, other than those usually considered, should be explored to find one that relates to both of these abnormalities. In the absence of a more complete understanding of the various functions of vitamin B_{12} that might control these seemingly divergent metabolic pathways, suggestions made here are clearly conjectural. An additional difficulty preventing a clear recognition of the mechanisms is the lack of uniform involvement of all epidermal cells. Such variation in involvement implies that cellular susceptibility to the effects of the deficiency must differ. This difference may reflect some inherent variation in cellular metabolism.

Jocelyn[32] demonstrated that a diminished level of reduced glutathione (GSH) is present in the red blood cells of patients with severe pernicious anemia. O'Dell et al[33] subsequently demonstrated a decreased level of nonprotein sulfhydryl (NPSH) with an associated increased nonprotein disulfide (NPSS) in the livers of vitamin B_{12}-deficient rats. He concluded that a shift from the reduced sulfhydryl (SH) to the oxidized disulfide (SS) occurs in vitamin B_{12} deficiency. Nonprotein (acid soluble) sulfhydryl has been found to be almost entirely GSH.[34] These data, though far from conclusive, suggest that a lack of vitamin B_{12} results in a decrease in the intracellular reduction potential or a shift in the SH $\rightleftarrows$ SS equilibrium to the right. This lowering of reduction potential may be due to a decreased amount of GSH, or the diminished GSH may simply be a reflection of an altered overall redox balance.[35]

Melanogenesis and Sulfhydryl Metabolism.—Melanin production depends on successive oxidative reactions beginning with tyrosine in the presence of the enzyme tyrosinase.[36] Tyrosinase is the rate-limiting enzyme and is normally inhibited by a sulfhydryl compound, probably GSH.[37] Removal or oxidation of glutathione has been shown to precede many states of hyperpigmentation.[37-39] Glutathione reductase acts in conjunction with reduced nicotinamide adenine dinucleotide phosphate (NADPH) to reduce oxidized glutathione (GSSG) to GSH (Fig 6). This enzyme may play a major role in regulating the more prolonged or the permanent changes in pigmentation by decreasing the cell's capacity to convert GSSG to GSH. Such a difference in glutathione reductase activity between black and white skin has been demonstrated by Halprin and Ohkawara.[40]

Mitosis and Sulfhydryl Metabolism.—Normal cellular growth and division are associated with reciprocal variations in the level of reduced SH and oxidized SS sulfhydryls.[41] Intracellular sulfhydryl content fol-

lows a cyclic fluctuation related to cell division reaching a maximum just before division occurs.[35,41] Such evidence has led to the proposal that the intracellular GSH ⇄ GSSG or glutathione cycle represents a mitosis-initiating and coordinating system. Factors causing a shift to the left seem to promote mitosis and those pushing the reaction to the right retard mitosis.[41] In addition, this system may also have a regulatory function due to its effect on various SH-dependent enzyme systems. Such enzymes may be switched off or on by the appropriate alteration of the redox balance.[42]

DNA and Sulfhydryl Metabolism.—Potent and specific sulfhydryl oxidizing agents have recently become available.[43] These agents can produce rapid, oxygen-independent oxidation of GSH to GSSG without impairing other vital metabolic functions of the cell. Experimental evidence with such agents confirms the expected growth inhibitory effect of GSH oxidation. In addition, oxidation of GSH in Ehrlich ascites tumor cells is accompanied by an immediate decrease in DNA synthesis, suggesting that impairment of DNA synthesis may occur as a result of a diminished GSH level.[44] Since impairment of DNA synthesis is widely accepted as the common denominator of the megaloblastic anemias and, as mentioned, megaloblastic anemias, due to a deficiency of vitamin B_{12}, seem to have a decreased intracellular GSH level, it is possible that the impairment of DNA synthesis that occurs in vitamin B_{12} deficiency might be secondary to a decreased availability of GSH.

Studies[45] of the relation between DNA and sulfhydryl metabolism have shown that the exposure of germinating seeds to SH-binding agents causes chromosomal aberrations. It is of interest that similar chromosomal changes occur in pernicious anemia and that these chromosomal abnormalities appear to result from the absence of vitamin B_{12} since they disappear following replacement therapy.[46-48] Such findings lend further support to the proposed relation between sulfhydryl, DNA, and vitamin B_{12} metabolism.

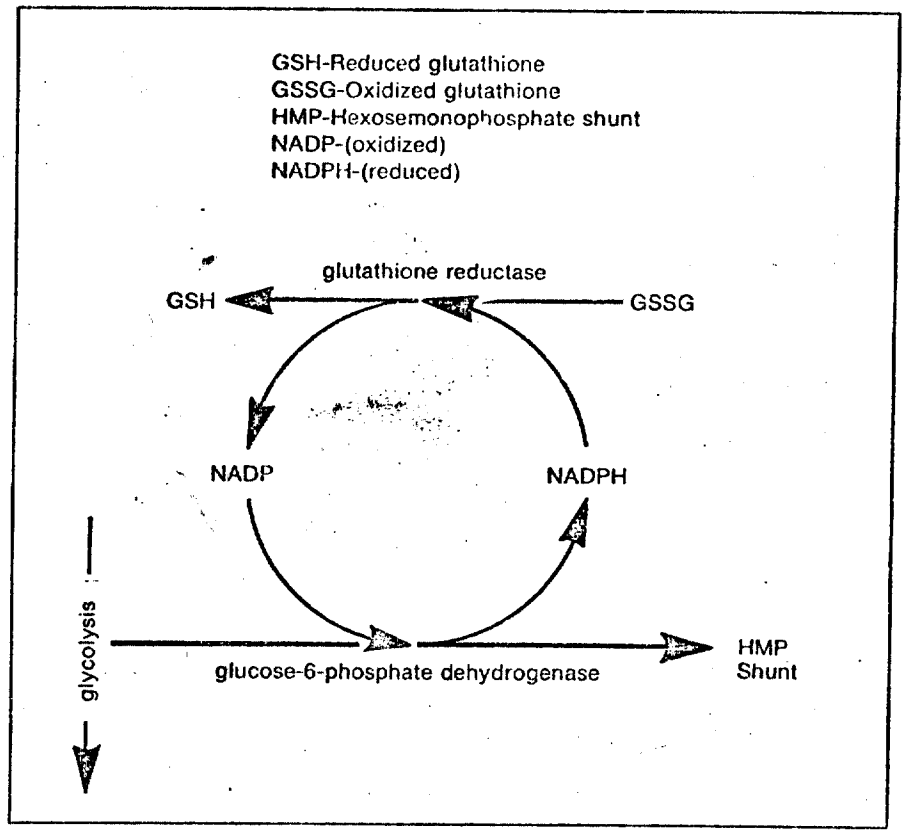

Fig 6.—Pathways relating to metabolism of glutathione.

Accordingly, it is suggested that a deficiency of vitamin B_{12} produces a lowering of the intracellular reduction potential, with a concomitant decrease in the GSH/GSSG ratio.[32,33,48] The epidermal melanocytes are then stimulated to produce melanin since the tyrosinase inhibiting effect of GSH has been diminished.[37] At the same time, the lowered GSH level results in retardation of mitosis, as well as diminished DNA synthesis.[35,41,43] This could result in epidermal atrophy and hyperpigmentation, as observed in our case. Kyle et al[49] described similar histologic changes in the skin of patients on long-term treatment with busulfan, which is a known sulfhydryl binding agent. Others have demonstrated widespread cytomegaly[50-52] and chromosome aberrations[53] in patients taking this drug.

It is possible that a better understanding of GSH metabolism might reveal a biochemical basis of various poikilodermatous skin conditions. Factors that may exert some influence on the synthesis, oxidation state, and utilization of glutathione are numerous.[33,40,41,52-63] Indeed, the GSH level may be modified by an almost unlimited variety of interrelated factors such as race,[41] nutritional status,[33,45,54,56] and metabolic[62] and endocrine[41,56,59] function. This may explain the extreme variation in response to exogenous agents tending to lower the intracellular GSH content, such as vitamin B_{12} deficiency, busulfan therapy, ultraviolet light,[38] or x-ray[41] exposure.

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Abnormalities of vitamin B_{12} and folic acid metabolism—their influence on the nervous system

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Recently Dr W. B. Castle of Harvard, one of the great figures of Medicine in this century, said, in the course of the first Sir Stanley Davidson Lecture of the University of Edinburgh, that little progress has been made in our knowledge of the causation of subacute combined degeneration of the cord, the neurological disorder that used to be such a striking complication of pernicious anaemia. Because of advances in therapy the condition is seldom seen now, but, despite all the progress that has been made in various fields of study of the megaloblastic anaemias, there

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has not been a parallel increase in our knowledge of the precise cause of the neurological complications.

Causes of depletion of vitamin B_{12} or folic acid

First let me remind you that megaloblastic anaemia occurs when there is a considerable degree of deficiency of vitamin B_{12} or folic acid or both, but that such deficiency states cause widespread metabolic upsets throughout the body, the abnormalities by no means being limited to the bone marrow.

Vitamin B_{12} deficiency arises from the following main causes:

(a) Dietary deficiency, as in vegans.

(b) Lack of intrinsic factor (pernicious anaemia, partial or total gastrectomy, rarely after gastroenterostomy).

(c) Malabsorption in any of the 'sprue' group of diseases, or from organic disease of the lower ileum or its surgical removal.

(d) Bacteria in a blind loop or stagnant area of small intestine directly or indirectly depriving the patient of vitamin B_{12}. The fish tapeworm, *Diphyllobothrium latum*, a parasite in the intestine of people living on the shores of the Baltic, also does this.

Subacute combined degeneration of the cord has been reported most consistently in association with Addisonian pernicious anaemia, but it may occur in any condition that leads to vitamin B_{12} depletion (Woltman & Heck, 1937; Badenoch, 1954; Wokes, 1956; Harrison, Booth & Mollin, 1956; Richmond & Davidson, 1958; Bourne & Oleesky, 1960; Riley, 1966).

Folic acid depletion is found as a result of:

(a) Dietary deficiency. This is rarely seen in the United Kingdom other than in pregnancy, but may be found in geriatric patients, particularly in industrial areas.

(b) Malabsorption because of one of the 'sprue' group of diseases, or from extensive organic disease of the small intestine.

(c) Metabolic competition for folic acid from certain anticonvulsant drugs or from folic acid antagonists.

(d) Increased need for folic acid as in pregnancy, or from haemolysis, prolonged infection or widespread malignancy.

It is usually considered that folic acid deficiency by itself does not lead to subacute combined degeneration of the cord, but there have been reports suggesting that neurological disorders can be associated with depletion of folic acid alone (Hansen, Nordqvist & Sourander, 1964; Anand, 1964; Grant, Hoffbrand & Wells, 1965). Such reports are, however, few and usually not very convincing. The most interesting examples are seven patients reported by Grant *et al.* (1965) who came to a neurological clinic with peripheral neuropathy or spastic paraplegia and were found to have folate depletion (but with higher serum folate levels than are usually found in severe folate deficiency). It is possible that the neurological disorder led to folate deficiency from impaired appetites or feeding habits, and certainly there is no evidence that folate deficiency causes subacute combined degeneration of the cord.

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Metabolic actions of folic acid and vitamin B_{12}

The precise metabolic activities of folic acid and vitamin B_{12} are not fully understood, and are a matter of great complexity. Much of what is believed to occur in man has been deduced from investigation carried out with bacteria or in animals, and, particularly as regards vitamin B_{12}, our knowledge is fragmentary.

Folic acid. There is reason to believe that in the cells of the body there is a 'pool' of 'one-carbon units' which are available for the synthesis of various substances. Forms of folate, called tetrahydrofolates because they contain four additional atoms of hydrogen, act as coenzymes. They receive single carbon units from the body pool and can donate them for the formation of various basic substances. The forms of tetrahydrofolate involved are the methyl, hydroxymethyl, methenyl, methylene, formyl and formimino derivatives. Cells containing folate have an enzyme system, named folic acid reductase, which permits reduction of folate to the tetrahydrofolate state.

The story is a very complicated one (Girdwood, 1959), but, in brief, derivatives of folic acid are involved in the formation of methionine from homocysteine and in the construction of purines and pyrimidines and hence of RNA and DNA. Thus folic acid is of fundamental importance in normal cell division and growth.

Vitamin B_{12}. So far as vitamin B_{12} is concerned, this substance has been found in micro-organisms and mammalian systems to be involved in the methylation of homocysteine to methionine, so that both it and folic acid are involved in methionine formation. There appear, however, to be a number of reaction mechanisms for vitamin B_{12}. In certain systems it is involved in isomerization reactions and, in others, in oxidation–reduction reactions. Unfortunately, too, the vitamin appears to carry out different functions in different organisms, and there are various active forms. Thus there is probably a methyl form which, in rats, is involved in the enzymatic methylation of soluble RNA to give methyl RNA (Venkataraman, Walerych & Johnson, 1967), and a dimethyl-benzimidazolylcobamide complex is possibly the storage form in man. ?

In vitamin B_{12} deficiency in man there is an increased urinary excretion of methylmalonate, because methylmalonyl coenzyme A is isomerized by a vitamin B_{12}-mediated reaction into succinyl coenzme A. In lactobacilli, and perhaps in man, ribotides are converted to deoxyribotides by a vitamin B_{12}-mediated reaction. We do not, however, know a great deal about the functions of vitamin B_{12} in man, and so far as the metabolism of nervous tissue is concerned our ignorance in this respect is almost complete. It was suggested some years ago by Wokes (1956) that when there is a lack of vitamin B_{12}, the sulphydryl system is inhibited and this leads to abnormal metabolism of tryptophan and tyrosine with consequent accumulation of toxic products. Weaver & Neill (1954) reported that there is excessive urinary excretion of amino acids and taurine in pernicious anaemia, and Swendseid, Wandruff & Bethel (1947 *a,b*) and Abbot & James (1950) suggested that interference with the normal metabolism of tyrosine and tryptophan could lead to an

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excessive excretion of phenolic bodies. Whether this has anything to do with neurological changes is by no means clear.

Findings in the cerebrospinal fluid

From time to time various workers have sought to investigate the neurological complications of vitamin B_{12} deficiency and other disorders by estimating the content of vitamin B_{12} or folic acid in the cerebrospinal fluid (CSF) (Herbert & Zalusky, 1961; Worm-Petersen, 1962; Gjertsen & Schrumpf, 1962; Simpson, 1964; Herbert, 1964; Wells, 1965). There have been certain discrepancies, but most authors agree that both in normal persons and in those with vitamin B_{12} deficiency the vitamin B_{12} level of the CSF is considerably less than that of the serum, whereas the folate level of the CSF is two or three times that of the serum, even in folate deficiency. A very recent paper (Wells & Casey, 1967) has shown a positive correlation between the *Lactobacillus casei* folate activities of serum and CSF in hospital control patients. This is not a subject that I have pursued very actively, but in Table 1 there are given the CSF vitamin B_{12} levels in a number of non-anaemic hospital patients whose serum vitamin B_{12} levels were within the normal range of 150–800 pg/ml.

Table 1. *Vitamin B_{12} levels in the cerebrospinal fluid of seventeen non-anaemic hospital patients*

Method of determination	Vitamin B_{12} concentration (pg/ml)								
Euglena gracilis	3	6	7	9	10	14	14	20	
Lactobacillus leichmannii	N	N	10	10	13	14	17	20	27

N, negligible.

The values in Table 1 are only approximate since, particularly with *L. leichmannii*, it is not possible to measure such small concentrations with any degree of precision. I wondered whether the low levels might indicate the presence of some agent that inhibited the growth of the test organism, but efforts to dilute out a hypothetical inhibitor were quite unsuccessful.

Pathological changes in the central nervous system in vitamin B_{12} deficiency

Time does not permit me to deal with this, but the changes in the spinal cord should no longer be seen by pathologists because the condition should not develop. It is one of patchy degeneration in the white matter of the posterior and lateral columns. First there is swelling of the medullary sheaths, then fatty degeneration and disappearance of the axis cylinders. There is, conspicuously, an absence of neuroglial proliferation.

Clinical problems

As I see it, the clinical problems related to the nervous system that require consideration are:

Peripheral neuropathy. This may occur in association with any anaemia of moderate or severe degree and may cause loss of vibration sense which, however, is not extensive and responds to antianaemic therapy. Paraesthesiae may be a feature.

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Posterior column degeneration. This may occur from deficiency of vitamin B_{12}. Paraesthesiae are a prominent feature, and there is pronounced loss of vibration sense, particularly in the lower limbs. Position sense is impaired, and there is likely to be peripheral neuropathy, depression of knee and ankle jerks and perhaps some loss of motor power.

Postero-lateral degeneration. This is now seldom seen in vitamin B_{12} deficiency, but, when it occurs, there is, in addition to the above features, spasticity and ataxia; extensor plantar responses are likely to be found. Postero-lateral degeneration may occur without anaemia, particularly if a diet with a high folate content is taken or, of course, if folic acid is given. It is not uncommonly found in vegans, and a possible instance of nutritional origin has been reported recently (Riley, 1966). There have been two reports of the condition progressing despite treatment with vitamin B_{12} (Ellis, Breur, Owen & Laszlo, 1966; Riley, 1967), but in both cases the patient had intestinal malabsorption in addition to possible pernicous anaemia, so the story is a complicated one.

I should perhaps at this point refer to a paper by Cox (1962) who reported on the findings in 478 patients with untreated pernicious anaemia--a reasonable number to merit consideration. Twelve had only mental disturbances and no other neurological features. Of the remaining 466, 39% had no neurological features, 23% had paraesthesiae, 12% had posterior column signs, and 26% had evidence of postero-lateral degeneration. In contrast, Davidson (1957) reported paraesthesiae in 38% of 250 patients, ataxia in 6% and spasticity in 1%, while Cabot (1908) found paraesthesiae in 100% of 1200 patients, ataxia in 7% and spasticity in 4%.

Psychiatric disorders in vitamin B_{12} depletion. When folic acid was first introduced as a haemopoietic agent and its inability to control the neuro-psychiatric features of pernicious anaemia was not realized, I gave it to six patients who showed a gratifying haematological improvement, but one attempted to murder his wife!

The mental abnormalities of vitamin B_{12} deficiency can occur without anaemia (McAlpine, 1929; Strachan & Henderson, 1965), and possible features include confusion, forgetfulness, intellectual deterioration, delusions, paranoia, mania, incontinence and possibly epileptiform attacks. There has been controversy about the best way to screen psychiatric patients for vitamin B_{12} deficiency (Henderson, Strachan, Beck, Dawson & Daniel, 1966; Hansen, Rafaelson & Rodbro, 1966; Varadi, 1966), but a recent prospective controlled investigation suggests that routine screening is not necessary (Shulman, 1967). The number studied was, however, small, and it is certainly important to remember that mental change in a relative of a patient with pernicious anaemia may be due to vitamin B_{12} deficiency. Diffuse abnormalities may be found in the electroencephalograms of vegans, but, unlike those of pernicious anaemia, the abnormalities are not corrected by treatment with cyanocobalamin (West & Ellis, 1966).

Psychiatric disorders in folate depletion. This has not been accepted as a feature in pregnancy or malabsorption, but was reported by a normal subject himself suffering from experimental folate deficiency, as consisting of sleeplessness, forgetfulness and irritability (Herbert, 1962). It has also been described in elderly people suffering

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from folate depletion (Strachan & Henderson, 1967), but there are problems of complicating arteriosclerosis and of the difficulties involved in confirming folate deficiency (Girdwood, Thomson & Williamson, 1967). It seems that in some areas of Great Britain folate deficiency in the aged is a considerable problem (Varadi & Elwis, 1967), whereas in others it is not, possibly because of differing dietetic habits. Nevertheless, it must always be borne in mind when an elderly person becomes disorientated or demented.

Possible neurological changes in folate depletion. Reference has already been made (p. 102) to the rarity of this.

Optic atrophy and retrobulbar neuritis. In deficiency of vitamin B_{12} or folic acid there may occur haemorrhagic retinopathy, impairment of visual acuity or disturbance of colour vision (Adams, Chalmers, Foulds & Withey, 1967; Gorrell, 1967). In addition, however, Heaton, McCormick & Freeman (1958), Heaton (1960) and Freeman & Heaton (1961) showed that the mean serum vitamin B_{12} level was reduced in tobacco amblyopia and suggested that the optic neuritis which is sometimes found in pernicious anaemia is tobacco amblyopia. Smith (1961) suggested that the neurological lesion in 'tobacco amblyopia' is due to chronic cyanide intoxication from tobacco smoke but it can occur in non-smokers (Adams *et al.* 1967), possibly from another source of cyanide. The condition is not related to the other neurological complications of vitamin B_{12} depletion. It is logical and more effective to treat 'tobacco amblyopia' or retrobulbar neuritis of vitamin B_{12} deficiency with hydroxocobalamin rather than cyanocobalamin (Chisholm, Bronte-Stewart & Foulds, 1967). It is possible that optic atrophy in prisoners of war in Japanese POW camps was due to vitamin B_{12} deficiency together with smoking, but although I saw many of these prisoners (Girdwood, 1950) I did not ask them about smoking habits.

Anticonvulsant drugs and megaloblastic anaemia. Finally, I must remind you that there may be megaloblastic anaemia responding to folic acid in patients under treatment with anticonvulsants, particularly primidone or phenytoin. Moreover, phenytoin is said to be neurotoxic (Utterback, 1958). The serum folate level is frequently low in patients treated with anticonvulsants, but there may be no significant difference between the levels in those with normoblastic and those with megaloblastic marrows (Reynolds, Milner, Matthews & Chanarin, 1966). The CSF folate level may also be low in drug-treated epileptic patients (Wells & Casey, 1967), but the significance of all this is uncertain.

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Separation of Transcobalamins on Small DEAE-cellulose Columns (36451)

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Three vitamin B_{12} binders have been isolated from normal serum by DEAE-cellulose column chromatography utilizing 3 × 60 cm columns (1–3). These binders have been designated as transcobalamin II (TCII), main protein peak binder (MPPB), and transcobalamin I (TCI) (2, 3). TCII has a molecular size of approximately 36,000, disappears rapidly from the circulation following intravenous injection into normal human subjects, and is bound to rat liver when perfused through the isolated organ (3, 4). MPPB has a molecular size of about 120,000 and acts like TCII when injected intravenously into human subjects or when it is perfused through the isolated rat liver (3, 4). TCI has a molecular size of about 121,000, but differs from TCII and TCI in that clearance from plasma is slow following intravenous injection into normal human subjects, and is poorly bound to isolated rat liver when perfused through the organ (3, 4).

In the present study DEAE-cellulose column chromatographic technique was adapted to small scale separation of vitamin B_{12} binders in 1 ml of serum.

Materials and Methods. Preparation of the adsorbent. The procedure has been described elsewhere (1). Forty grams of dry DEAE-cellulose (Schleicher and Schuell, No. 70, 0.90-0.94 mEq/g) were allowed to sink in 2 liters of 1 *N* NaOH. After 30 min, suspension was filtered through a coarse fritted-glass filter funnel and washed with additional 1 *N* NaOH until filtrate was colorless. Filter cake was suspended in distilled water, filtered through the funnel, and washed with distilled water to pH 7.0. The cake was resuspended in 4 vol of 0.0175 *M* sodium phosphate buffer (pH 6.3), and enough 0.0175 *M* solution of $NaH_2PO_4 \cdot H_2O$ added to bring the pH to 6.2. DEAE-cellulose was washed in funnel with 0.0175 *M* sodium phosphate buffer (pH 6.3) until pH of suspension was 6.3. Cellulose was transferred to a beaker where it was resuspended in sufficient buffer to make about 50 ml of suspension for each gram of dry cellulose used.

Suspension was packed into 0.6 × 25 cm columns (5 ml pipettes plugged with glass wool). Columns were equilibrated overnight with 70 ml of 0.0175 *M* sodium phosphate buffer (pH 6.3) at room temperature. Tubing connecting the buffer reservoir (250 ml separatory funnel) with column was at a lower level than tip of column, so that column remained moist.

Preparation of the serum. Serum was kept frozen at —20°. The following quantities of ^{57}Co B_{12} (Amersham, sp act ca. 100 $\mu Ci/\mu g$) were added per milliliter of thawed serum: 250, 500, 1000, 2500 and 5000 pg. Radioactivity was counted in a well-type scintillation spectrometer. Solution was allowed to stand at 37° for 15 min and transferred into dialysis tubing. Residual activity in test tube was counted. Labeled serum was dialyzed at 4° against an excess of 0.0175 *M* buffer (pH 6.3) for 24 to 48 hr. At the end of this period dialysis tubing was placed in a 16 × 150 mm test tube and nondialyzable activity was recorded.

Chromatographic procedure. Any solution remaining above the column after equilibration with initial buffer was allowed to sink into adsorbent and serum sample pipetted onto the surface. Test tubes, 13 × 100 mm, marked to 2 ml volume, were used for collection of eluate. Residual activities in test tube and dialysis tubing were counted for radioactivity. When entire sample entered adsorb-

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TABLE I. Chromatographic Separation of Normal Sera (10 Persons) and CML Sera (2 Patients) Following Addition of Indicated Amount of $^{57}Co\ B_{12}$ per Milliliter of Serum.

Added $^{57}Co\ B_{12}$ (pg): Fraction	250	500	1000	2500	5000
		Normals (%)			
Bound	82 ± 6[a]	85 ± 9	83 ± 9	51 ± 17	29 ± 5
TCII	70 ± 8	68 ± 7	70 ± 7	70 ± 8	69 ± 7
MPPB	23 ± 8	24 ± 7	23 ± 6	22 ± 6	23 ± 6
TCI	7 ± 2	8 ± 1	7 ± 2	8 ± 2	8 ± 1
Recovery	89 ± 3	87 ± 9	85 ± 9	92 ± 5	89 ± 5
		Patients[b] (%)			
Bound	99	93	91	99	95
	99	99	99	99	99
TCII	12	12	12	12	12
	6	5	5	6	6
MPPB	44	52	46	45	54
	37	32	49	43	40
TCI	44	36	42	43	34
	57	63	46	51	54
Recovery	86	91	92	74	90
	77	83	87	78	77

[a] SD of the mean.
[b] Top line, patient H.; second line patient G.

ent, top of column was rinsed with 3 small portions of 0.0175 *M* buffer. Thereupon empty part of tube was filled with the initial buffer and the column was connected to a 250 ml separatory funnel containing 0.0175 *M* sodium phosphate buffer (pH 6.3). Six 2 ml samples were collected with this buffer. After sixth 2 ml sample was obtained, buffer reservoir was quickly disconnected and a new one containing 0.04 *M* sodium phosphate buffer (pH 5.9) was used for elution of twenty 2 ml samples. In same manner six 2 ml samples were collected with 0.1 *M* sodium phosphate buffer (pH 5.8), and seven 2 ml samples with 0.4 *M* sodium phosphate buffer (pH 5.2). Buffers were prepared in distilled water containing 0.09% methylparaben and 0.01% propylaraben (1). Tubes containing eluate were counted for radioactivity. TCII was eluted with the 0.04 *M* sodium phosphate buffer (pH 5.9) (1, 3); MPPB with 0.1 *M* sodium phosphate buffer (pH 5.8) (1, 3); TCI with 0.4 *M* sodium phosphate buffer (pH 5.2) (1, 3). Any free $^{57}Co\ B_{12}$ present in the serum was eluted with 0.0175 *M* sodium phosphate buffer (pH 6.3).

Results and Discussion. Table I indicates results obtained from separation of vitamin B_{12} binders in serum of 10 normal persons. The percentage bound $^{57}Co\ B_{12}$ was practically constant for amounts of added $^{57}Co\ B_{12}$ up to 1000 pg/ml. At 2500 and 5000 pg/ml levels of added $^{57}Co\ B_{12}$ percentage bound vitamin decreased, although absolute amount bound increased. Percentage of $^{57}Co\ B_{12}$ bound to each of three binders remained essentially constant for quantities of added vitamin B_{12} used in this study. Significantly more $^{57}Co\ B_{12}$ was bound to MPPB than to TCI in all 10 sera.

Table I indicates results obtained from separation of the binders in serum of two patients with chronic myelocytic leukemia. Percentage bound $^{57}Co\ B_{12}$ was increased. The percentage of $^{57}Co\ B_{12}$ bound to TCII decreased significantly in both cases with a corresponding increase in activity bound to MPPB and TCI. This disease is evidently associated with a significant increase of MPPB and TCI in the serum. Because of similarity in molecular size of MPPB and TCI (3), gel filtration does not separate these two binders and results obtained by this method show a high TCI content. Since

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TCII has the tendency to aggregate (5), it is possible that MPPB is an aggregate of TCII. Conditions under which TCII undergoes aggregation are not well understood.

It thus appears that MPPB is normally present in human serum and can be separated from other B_{12} binders by DEAE-cellulose column chromatography, provided a suitable elution system is used.

Summary. A DEAE-cellulose column chromatographic technique was used for separation of TCII, MPPB and TCI in 1 ml of human serum. The percentage of vitamin bound to each binder remained practically constant at levels of added vitamin varying from 250 to 5000 pg ^{57}Co B_{12}/ml of serum. The percentage ^{57}Co B_{12} bound to MPPB and TCI in chronic myelocytic leukemia serum was significantly higher than in normal serum.

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BLOOD SERUM LEVELS AND INTESTINAL ABSORPTION OF VITAMIN B_{12} IN MULTIPLE SCLEROSIS

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In view of the conflicting data in the literature as to the coexistence of B_{12} deficiency in multiple sclerosis, the authors determined the B_{12} blood serum levels, by the Ochromonas malhamensis technique, and the intestinal absorption of radioactive vitamin B_{12}, by the hepatic uptake method, in twenty-one cases of this disease. This study failed to detect any significant B_{12} deficiency or derangement of intestinal absorption of vitamin B_{12} in multiple sclerosis.

THE pathology of multiple sclerosis is characterized by the formation of demyelinated glial plaques, with naked axis cylinders and virtually intact ganglion cells, in the white matter of the central nervous system.[1] Since demyelination of the long posterior nerve axons is a typical pathologic finding in severe vitamin B_{12} deficiency,[2] several investigators have studied the correlation between multiple sclerosis and the neuropathies found in vitamin deficiencies.[3-6] Vitamin B_{12} is known to perform many physiologic roles in normal nerve metabolism by virtue of its participation in the synthesis of ribose nucleic acid and the transmethylation reactions.[7] The latter are important for the formation and preservation of choline, an integral part of the molecules of lecithin and sphingomyelin contained in the myelin sheaths of nervous tissue.[2, 8-11]

The data available in the literature dealing with the role of vitamin B_{12} deficiency in multiple sclerosis tend to conflict, however. While Sobotka, Christoff, and Baker,[12] using the *Ochromonas malhamensis* assay, found the B_{12} levels in the blood serum and cerebrospinal fluid elevated in about one-third of their twenty cases of multiple sclerosis, O'Connor and co-workers,[13] using the *Lactobacillus leichmannii* assay, found, on the contrary, low B_{12} blood serum in ten of their twenty-one cases of multiple sclerosis, the levels falling to within the range found in patients with pernicious anemia. A similar conflict in results was found when vitamin B_{12} was used for the treatment of multiple sclerosis.[1, 3-6, 14] While one group of investigators reported marked improvement in cases of multiple sclerosis following parenteral treatment with vitamin B_{12},[3, 4] others were unable to duplicate these findings.[1, 5, 6]

In the light of these conflicting views as to the role of B_{12} in the pathogenesis of multiple sclerosis, this investigation was undertaken.

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MATERIALS AND METHODS

A total of twenty-two cases of demyelinating neurological disease were studied, twenty-one of which were clinically diagnosed as multiple sclerosis. All patients had a thorough neurological evaluation. Some additional detailed information on their status is listed in Table I.* The vitamin B_{12} blood serum levels were determined in all cases by *Ochromonas malhamensis* assay[15] as modified by Hutner and associates.[16] In three of the multiple sclerosis cases, these levels were determined, in addition, by the *Euglena gracilis* method.[17]†

VITAMIN B_{12} IN BLOOD SERUM AND HEPATIC UPTAKE OF RADIOACTIVE VITAMIN B_{12} IN 22 CASES OF MULTIPLE SCLEROSIS.

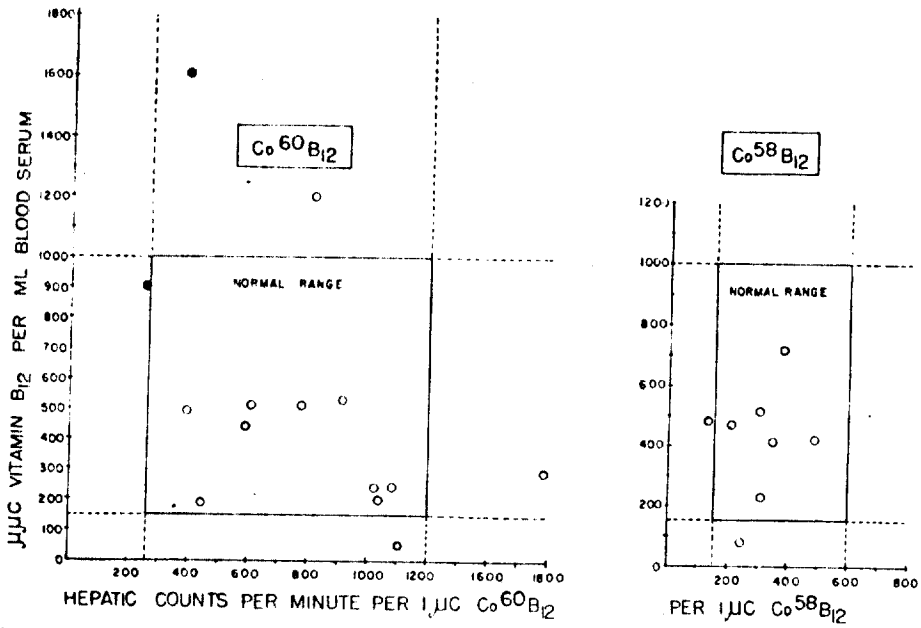

Fig. 1.—Vitamin B_{12} in blood serum and hepatic uptake of radioactive vitamin B_{12} in 22 cases of demyelinating neurologic disease, including 21 cases of multiple sclerosis.

In all twenty-two cases, intestinal absorption of vitamin B_{12} was determined by measurement of the hepatic uptake of radioactive B_{12}.[18] Counts over the liver, with control counts over the intestines, were taken on the sixth, seventh, and eighth days following oral administration of labeled vitamin B_{12}. For this purpose, a scintillation counter with no collimator was used, with a NaI thallium crystal (1 by ¾ inch), at a 1,100 volt setting. Three hepatic and 2 intestinal control projections were counted. The uptake of radioactivity over the liver was averaged from the 3 projections, calculated above background, corrected for intrinsic error of the scaler and for physical decay of the material, with the use of labeled B_{12} standards, and expressed per 1 μc of radioactive cobalt ingested. Seventeen patients received $Co^{60}B_{12}$,‡ fifteen at a dose of 0.46 to 0.48 μg (containing 0.13 μc Co^{60}), and two at a dose of 4.83 μg (containing 0.576 μc Co^{60}). Eight patients received $Co^{58}B_{12}$‡ at a dose of 0.598 μg (containing 2.91 to 2.93 μc Co^{58}).

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‡Radioactive vitamin B_{12} was supplied by Dr. Nathaniel S. Ritter and Dr. Charles Rosenblum of the Merck Sharp & Dohme Research Laboratories, Merck & Co., Inc., Rahway, N. J.

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TABLE I. VITAMIN B_{12} IN BLOOD SERUM AND HEPATIC UPTAKE OF RADIOACTIVE B_{12} IN TWENTY-TWO CASES OF DEMYELINATING DISEASE

NO.	PATIENT	SEX	AGE (IN YEARS)	NUTRITIONAL STATUS	LAST HEMOGLOBIN (GM. %)	LAST HEMATOCRIT (%)	PREVIOUS TREATMENT WITH VITAMIN B_{12}*	VITAMIN B_{12} IN BLOOD SERUM $\mu\mu g$/ML.		HEPATIC UPTAKE OF RADIOACTIVE B_{12}		
								OCHROMONAS MALHAMENSIS ASSAY	EUGLENA GRACILIS ASSAY	TRACER	DOSE	HEPATIC UPTAKE (CPM/1 μC)
1.	R. G.	M	28	Poor	12.4	42	100 μg I.M. daily for 1 month, 5 months prior to assay	721		Co^{58} B_{12}	0.598 μg = 2.9 μc	385
2.	C. I.	M	30	Poor	13.0		100 μg I.M. daily for 3 weeks, 5 months prior to assay	512		Co^{58} B_{12}	0.598 μg = 2.9 μc	316
3.	F. H.	M	59	Good	14.4			228		Co^{58} B_{12}	0.598 μg = 2.9 μc	312
4.	P. McG.	M	47	Good	14.9			423		Co^{58} B_{12}	0.598 μg = 2.9 μc	485
5.	D. B.	M	51	Good	15.0	43	100 μg I.M. daily for 3 weeks, 4 months prior to assay	479		Co^{58} B_{12}	0.598 μg = 2.9 μc	136
6.	S. E.	M	54	Good	14.4			467		Co^{58} B_{12}	0.598 μg = 2.9 μc	212
7.	H. C.	M	56	Fair	13.6	39	30 μg I.M. weekly for 5 weeks, 2½ years prior to assay	416		Co^{58} B_{12}	0.598 μg = 2.9 μc	348
8.	A. K.†	M	75	Good	12.3	39	Some treatment 2 years before	82		Co^{58} B_{12}	0.598 μg = 2.9 μc	251
9.	R. D.	F	45	Fair	12.8	41		239		Co^{60} B_{12}	0.48 μg = 0.43 μc	1,082
10.	I. F.	F	51	Obese	12.4	41		439		Co^{60} B_{12}	0.48 μg = 0.43 μc	595
11.	E. J.	F	43	Good	12.9	38	200 μg I.M. 3 times weekly for 3 months, ending 2 months prior to assay	1,205		Co^{60} B_{12}	0.48 μg = 0.43 μc	850
12.	J. K.	F	52	Obese	12.7	44		492		Co^{60} B_{12}	0.48 μg = 0.43 μc	394
13.	H. E.	F	59	Good			Some parenteral treatment, discontinued 9 months prior to assay	289		Co^{60} B_{12}	0.48 μg = 0.43 μc	1,590

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14.	L. S.	F	49	Poor	12.4			192		Co^{60} B_{12}	0.48 μg = 0.43 μc	445
15.	J. M.	F	38	Poor	13.6	42	30 to 100 μg I.M. 2 or 3 times per week for 4 months, ending 1 month prior to assay	1,608			4.8 μg = 0.58 μc	380‡
16.	T. L.	F	30	Poor	11.5	39		900		Co^{60} B_{12}	4.8 μg = 0.58 μc	255‡
17.	H. B.	M	57	Good	14.7	54		50	95	Co^{60} B_{12}	0.46 μg = 0.43 μc	1,112
18.	L. W.	F	36	Poor	12.1	44		534		Co^{60} B_{12}	0.46 μg = 0.43 μc	915
19.	P. O.	F	58	Fair	13.0	40		505		Co^{60} B_{12}	0.46 μg = 0.43 μc	611
20.	M. M.	F	39	Good	14.3	48		505		Co^{60} B_{12}	0.46 μg = 0.43 μc	780
21.	J. K.	M	54	Good	16.0	49		(1) 417 (2) 241	516 190	Co^{60} B_{12}	0.46 μg = 0.43 μc	1,024
22.	E. S.	M	67	Good	12.1	37		200	146	Co^{60} B_{12}	0.46 μg = 0.43 μc	1,042

*Blank spaces below indicate that no information is available concerning prior treatment with vitamin B_{12}.

†The diagnosis in this case was "disseminated degenerative disease of the cerebrospinal system."

‡The labeled B_{12} in these 2 tests was of low specific activity in that the labeled material was diluted with "cold" B_{12} in a ratio of about 1 to 10, and the total oral dose of B_{12} was increased to 4.8 μg. The normal efficiency of the intestinal absorption of B_{12} at this oral dose is only about one-third to one-fourth of that which occurs at the 0.5 μg level.[25, 26] To keep the hepatic uptake in the normal range under these circumstances, the intestinal absorption of vitamin B_{12} must have been very good in both these individuals. These 2 cases are indicated by black dots in Fig. 1.

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RESULTS

Results of assay of the vitamin B_{12} blood levels and measurement of the hepatic uptake of labeled vitamin B_{12} are shown in Fig. 1 and listed in Table I.

B_{12} Blood Serum Levels.— These were within normal limits, which for the *Ochromonas* method, lie between 150 and 800 $\mu\mu$g per milliliter,[19] in seventeen of the twenty-two patients. Three patients had levels above the normal, i.e., ranging from 900 to 1,608 $\mu\mu$g per milliliter. Two of the latter had previously had parenteral treatment with vitamin B_{12} (see Table I). Two patients had very low B_{12} blood levels, in the pernicious anemia range, i.e., 50 and 82 $\mu\mu$g per milliliter, respectively. One of these cases was diagnosed as multiple sclerosis, the other as degenerative disseminated demyelination disease with severe malnutrition. When assayed by *Euglena gracilis,* this multiple sclerosis case was also found to have exceedingly low B_{12} levels, i.e., 95 $\mu\mu$g per milliliter. In two other cases of multiple sclerosis B_{12} serum levels were in the lower range of the normal, i.e., 192 and 146 $\mu\mu$g per milliliter, respectively. Intestinal absorption of vitamin B_{12} was normal in all the four cases with low B_{12} blood levels if judged by the normal results of measurements of the hepatic uptake of radioactive B_{12} (see Table I).

Hepatic Uptake of Radioactive Vitamin B_{12}.—Following oral administration of a standard dose of labeled B_{12}, hepatic uptake of radioactive B_{12} was performed on all twenty-two cases. Nineteen of the twenty-two cases studied had an intestinal absorption of labeled B_{12} within normal range,[20] i.e., with our settings, 300 to 1,200 c.p.m. per microcurie of Co^{60} and 150 to 600 c.p.m. per microcurie of Co^{58}. One case had an increased absorption, i.e., 1,590 c.p.m. per microcurie of Co^{60}. Only two cases of multiple sclerosis showed a low intestinal absorption of vitamin B_{12}, as demonstrated by their hepatic uptake of 255 c.p.m. per microcurie of Co^{60} and 136 c.p.m. per microcurie of Co^{58}, respectively. The B_{12} blood serum levels of both these cases were within normal limits.

DISCUSSION

The results of our assays of vitamin B_{12} blood serum levels and the intestinal absorption of vitamin B_{12} indicate that the great majority of cases of multiple sclerosis do not present evidence of any significant vitamin B_{12} deficiency or vitamin B_{12} absorption defect.

Our data on the blood serum levels of vitamin B_{12} in patients with multiple sclerosis do not corroborate the previous findings of other authors.[12, 13] This cannot be explained by the prior vitamin B_{12} treatment, since, to our knowledge, this was given to only five of our patients within the year preceding this study (compare data in Table I). Our results indicate that B_{12} serum levels are, in the vast majority of cases, within the normal range. A few cases reported in the literature point to the same conclusion.[21, 22]

We found the intestinal absorption of vitamin B_{12} to be normal in most of our cases of multiple sclerosis. This is in agreement with the findings obtained by O'Connor and co-workers[13] with the use of the urinary excretion technique. Other authors also have reported isolated cases of multiple sclerosis and degenerative neurological disease with normal intestinal absorption of B_{12}.[23, 24]

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CONCLUSIONS

Investigation of the blood serum levels of vitamin B_{12} by the *Ochromonas malhamensis* method and study by the hepatic uptake method of the intestnial absorption of radioactive vitamin B_{12} in twenty-one cases of multiple sclerosis fail to indicate any significant vitamin B_{12} deficiency or B_{12} absorption defect in this disease.

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Biliary and Fecal Vit. B_{12} Excretion in Man. An Isotope Study.* (23879)

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In spite of considerable progress in Vit. B_{12} research, it is still not known what amount normal humans have to absorb in order to maintain health; likewise, the optimum maintenance dose for pernicious anemia patients is unknown. One way to elucidate these questions is to study quantitatively the excretion of Vit. B_{12}. As judged from the minimum maintenance dose for pernicious anemia patients, normal humans seem to absorb at least one microgram *pro die* under physiological circumstances(1). When pure Vit. B_{12} is given *per os*, the upper limit of absorption lies well above this figure(2,3). On the other hand

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normal humans excrete less than one microgram in the urine per day(4). This indicates that the vitamin is either broken down in the body or at least is excreted in urine as a microbiologically inactive compound, or, alternatively, is excreted mainly by routes other than the urine. Previous studies on animals(5,6) demonstrate the importance of the biliary and fecal routes in the excretion of "endogenous" (absorbed) B_{12}. Indications of the significance of these routes in man were obtained with the finding of high microbiological B_{12} activity in human bile(5,6). Also, as judged from a few observations reported in the literature(7,8,9), it seems that in man parenterally administered radiovitamin B_{12} appears in the bile and feces. However, in no case has this excretion been subject to a systematic study. Because of the relatively large doses administered, the short observation times, and the change in excretion pattern of radiovitamin during the first days after injection (Fig. 1), no definite conclusion can be drawn as regards the physiological excretion routes of Vit. B_{12}.

In the present study it was attempted to study systematically the biliary and fecal B_{12} excretion by administering parenterally minimal doses of radiovitamin B_{12} and by subsequently following for long periods its elimination with the bile and feces. It was felt that such an investigation could not be restricted to the fecal route alone, since the extensive synthesis and possible breakdown(10) of B_{12} taking place in the intestinal contents make it impossible to draw any conclusions as to whether the fecal radioactivity is derived from excreted Vit. B_{12}.

Material and methods. A single 0.5 μg dose of Co^{56} labelled Vit. B_{12} with a specific activity of approximately 2 mc/mg (the authors are greatly indebted to Dr. E. Lester Smith, Glaxo Ltd., England, for the generous gift of $Co^{56}B_{12}$) was injected intramuscularly to the following subjects: a) 5 healthy young adult females, whose feces were subsequently collected in one day portions for 26-29 days; 24-hour urine samples were collected the first 2 days after injection and thereafter at irregular intervals; b) 5 patients with a postoperative T-tube drain in the common bile duct and without clinical signs of disordered B_{12} metabolism and whose drain bile was collected in one day portions until the drainage was discontinued; c) 2 healthy young adult males, who were subjected to duodenal lavage 3 times during the 1st to 40th day after the injection. The duodenal lavage was performed in the usual manner by passing down the tube during X-ray fluoroscopy and by injecting magnesium sulfate through the tube. Before counting, the feces samples were liquefied by nitric acid digestion, and the bile and urine samples were concentrated by evaporation. In counting, scintillation technic was used. Since it is not possible, even by drainage, to collect the daily bile secretion quantitatively it was attempted to calculate the daily biliary Co^{56}-B_{12} excretion in the following manner: the bilirubin content of the bile samples was determined, and the radioactivity found was corrected assuming that the daily bilirubin excretion was 270 mg.†

Results. The excretion pattern changing markedly after the first few days following injection, the data have been grouped into an *early* (1st-4th day) and a *late* (4th-30th day) group (Fig. 1).

Urinary excretion. Even with the very small amounts of B_{12} administered in this study, considerable amounts of radioactivity are eliminated by this route during the first 2 days after injection. During the early period, more Vit. B_{12} is excreted by this route than by any other (Table I, Fig. 1). On the other hand, during the late period the urine contained less radioactivity than the feces. In contrast to the feces samples, which all contained demonstrable radioactivity, in some late urines no activity could be detected.

Fecal excretion. Although the values observed early after the injection are distinctly higher than later on, this effect is much less marked than in the urine (Table I, Fig. 1). During the late period, the mean 24-hour excretion of radioactivity varies relatively little

† This was calculated as follows: Normal total body hemoglobin is approx. 750 μg(20), corresponding to 29.0 g of heme. Calculating with a breakdown of 1/120/day, this corresponds to excretion of 230 mg of bilirubin. To this are added 40 mg from non-hemoglobin porphyrins(21,22).

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TABLE I. Daily Excretion of Radioactivity, Calculated as B_{12}, after Intramuscular Injection of 0.5 μg Co^{56}-B_{12}.

			mμg/day	Range	% excreted of total B_{12}-Co^{56} in body	No. patients	No. of 24-hr collections analyzed
Early (1st-4th day)	Bile	Observed	4.8 ± 1.4†	.6-10.5		7	18
		Calculated	13.2 ± 3.9	2.6-29.1		7	18
	Feces		4.6 ± 2.0	1.5-8.6		5	15
	Urine*		25.8 ± 2.4	12.9-32.9		5	10
Late (4th-30th day)	Bile	Observed	.7 ± .2	.4-1.6		7	19
		Calculated	5.1 ± 1.3	1.4-11.5	1.1 ± .3	7	19
	Feces		1.4 ± .1	1.1-1.7	.3 ± .02	5	123
	Urine		.1 ± .09	.0-.5	.02 ± .02	5	10

* Urine collected for 2 days only. † Mean ± S.E.

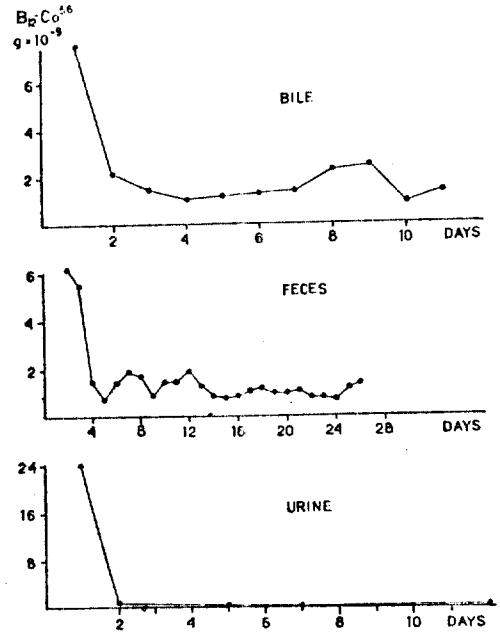

FIG. 1. Difference between *early* (1-4th day) and *late* (4th-30th day) period after parenteral administration of vit. B_{12}-Co^{56} as regards the pattern of radiovitamin excretion in 3 cases. Regarding excretion in bile and urine, those cases were chosen in which the largest number of samples were obtained. As regards fecal excretion, the case was chosen at random. There was considerable day-to-day variation, probably explained by differences in frequency of defecation and quantity of stools passed on different days. The curve represents successive mean values.

in different subjects. No feces samples were found to be nonradioactive. The average amount of radioactivity excreted by this route per day during the late period (calculated as Co^{56}-B_{12}) corresponds to 0.3% of the radioactivity present in the body (injected amount *minus* that excreted during the early period).

Biliary excretion. The early values are distinctly higher here also. The mean late excretion value is 5.1 mμg/day, corresponding to 1.1% of the amount of radiovitamin B_{12} present in the body after the early period (Table I).

The calculated corresponding excretions of "endogenous" vitamin are seen in Table II.

Discussion. The question naturally arises, whether the behavior of the injected radiovitamin is representative of the fate of the nonlabelled vitamin normally contained in the body.

The results of other workers(11-14) who have studied the distribution of radioactivity in different organs after oral or parenteral administration of radiovitamin B_{12}, indicate a

TABLE II. Daily Excretion of Endogenous Vitamin B_{12}. The calculations have been based on the assumption that the injected radiovitamin mixes with the body stores.

	Mean ± S.E.	Range
	μg	
Total amt of B_{12} in body*	3,900	790-11,100
Corresponding daily excretion of endogenous B_{12}		
Bile	43 ± 12	9-122
Feces	12 ± 1	2-33
Urine	.8 ± .8	.2-3

* Obtained by adding microbiological B_{12} contents of liver, kidneys, heart, spleen, brain, and muscles in 4-22 autopsies (varying for different organs)(15,16). The corresponding excretion of endogenous B_{12} is calculated using percentages given in Table I. Stand. errors are based only on isotope studies and do not include those of microbiological assays.

relatively good mixing of the introduced isotope with the body B_{12} pool. The distribution of radioactivity closely resembles the natural distribution of microbiological B_{12} activity (15,16).

Partly because this study deals with a human material it is difficult to establish definitely the time required for the complete mixing of the isotope among the body B_{12}-pool. In the present study the fourth day was chosen as the limit, because by this time the excretion pattern had undergone a marked change from the dominantly urinary excretion pattern into one characterized by a steady fecal output of radioactivity. In addition, other investigators(13,14) have found that by this time the distribution of parenterally injected radiovit. B_{12} has assumed the "final" pattern with maximal activity over the liver area. Although the percentage excretion of radioactivity does not seem to be much affected by the fixing of the limit to a few days later than the 4th day after the injection, the question on the mixing of injected radiovit. B_{12} has to be subjected to further study. Until such studies are concluded, the data given on the excretion of endogenous Vit. B_{12} should be interpreted with caution.

Irrespective of whether the mixing within the period of time studied in the present paper is absolutely complete, the conclusion seems well founded that in man Vit. B_{12}, or its cobalt containing breakdown product, is excreted mainly by the fecal route. The source of the fecal material may well be the bile, which was found to have relatively high radioactivity and which is rich in microbiological B_{12} activity(5,6). Our data indicate that at least two-thirds of the biliary radioactivity is reabsorbed from the intestine, and thus that Vit. B_{12} is subject to an enterohepatic circulation as are other components of the bile. It is possible that this reabsorption is aided by the gastric intrinsic factor and/or the Vit. B_{12} binding substance found to occur in bile(5,6). An impaired intestinal reabsorption may be a factor in the pathogenesis of certain B_{12} deficiency states. Preliminary results with simultaneous collection of bile and feces in the same subjects seem to confirm the concept of an intestinal reabsorption of Vit. B_{12}(17).

That absorbed Vit. B_{12} is excreted by the bile-feces route probably explains the fact that in performing fecal excretion tests, some radioactivity can be detected in the stools for a long time after the administration of the oral dose. Biliary excretion with subsequent isotope dilution in the intestine may explain the observed inhibition of radiovitamin B_{12} absorption by parenteral administration of nonlabeled B_{12}(18).

Provided the introduced radiovitamin is uniformly mixed with the nonlabeled vitamin contained in the body, it should be possible to calculate from the present data the corresponding excretion of endogenous B_{12} if the size of the B_{12} pool be known. Thus far tentative calculations have been performed, in which the size of the pool has been estimated on the basis of microbiological assays performed by other authors(15,16) on autopsy material. The results are listed in Table II. Even allowing for considerable errors, the data seem to indicate that the daily loss of Vit. B_{12} may amount to several micrograms, and thus the requirement of B_{12} may be larger than hitherto assumed(1). The order of the magnitude of the daily loss of Vit. B_{12} receives some support in recent investigations on the hepatic storage of radiovit. B_{12}(11,19), indicating a loss of several micrograms of radiovitamin per day.

Summary. It was attempted to study Vit. B_{12} excretion in man by investigating the elimination of small doses of parenterally administered radiovitamin Co^{56}-B_{12}. Relatively large amounts of radioactivity were excreted in the bile, feces and urine shortly after injection. After this period, the excretion pattern changed, and radioactivity of relatively constant value was excreted with the bile and feces, indicating that these are the main excretion routes in man, whereas the urine samples exhibited low radioactivity. The fecal radioactivity was only about one-third of the calculated biliary excretion, which indicates intestinal reabsorption of biliary B_{12}. If it were assumed, that the radiovitamin mixes uniformly with the body stores of Vit. B_{12}, it could be estimated that normal subjects may lose considerable amounts of B_{12} per day, and consequently, the requirement of

B_{12} could be larger than hitherto suspected. The findings may have therapeutical implications.

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THE BODY WEIGHT COURSE AND VITAMIN B_{12} ABSORPTION AFTER TRANSPOSITION OF THE JEJUNUM AND ILEUM IN THE RAT

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Abstract. A model for a study of the small intestine of the rat is presented. The positions of the proximal and distal parts of the small intestine were interchanged. The body weight course, the Hb and Hct values and the vitamin B_{12} absorption were studied and the results are presented. No distinct difference between these animals and intact controls was found with regard to the body weight changes and Hb and Hct values. No difference in the absorption of vitamin B_{12} was observed. The question of whether it is the duration of contact of the B_{12} intrinsic factor complex with the intestinal mucosa or the presence of specific receptors that is decisive for the location of B_{12} absorption is discussed.

Between the proximal and distal segments of the small intestine there are both morphological and functional differences. The borderline between these segments in this respect is not sharply delimited, however.

Kerckring's folds are most numerous, broadest and highest in the jejunum (Bockus, 1964). The same holds for the villi, which are most numerous and highest in the jejunum. The circular muscular layer is strongest in the proximal small intestine, the lymphoid tissue is most abundant in the ileum and the mesenteric vascular pattern most complex in the vicinity of the ileocaecal angle.

The propulsive motility is most rapid in the proximal small intestine. In the distal ileum the food has a long duration of contact with the absorption surface on account of the slow propulsive motility (Derblom, Johansson & Nylander, 1965).

Most investigators seem to agree that carbohydrates, after enzymatic disintegration, are absorbed mainly in the jejunum (Bockus; Booth, 1965). Protein and fat are also absorbed mainly in the jejunum but possibly a little more distally (Bockus; Booth). Borgström, Dahlqvist, Lundh & Sjövall (1957) found by means of an intubation technique in man that the fat absorption was completed in the first 50–100 cm of the jejunum.

Water-soluble vitamins and iron are also absorbed in the proximal part of the small intestine (Booth).

Vitamin B_{12}, on the other hand, according to unanimous reports, is absorbed distally in the small intestine. Booth states that B_{12} absorption is an active process that can only take place in the ileum and not in proximal sections of the intestine. Bockus considers that the absorption of vitamin B_{12}, both in man and experimental animals, occurs mainly in the middle and distal parts of the ileum and that there are specific receptors on the surface of the ileal mucosa for this absorption. There are some investigators, however, including Smith & Ellis (1965), who have not found such specificity in the ileum. These and other authors, for example Okuda (1960) have suggested that the motility conditions, with rapid propulsion in the jejunum and slow propulsion in the ileum, can be of importance for the B_{12} absorption in the ileum. The vitamin B_{12} intrinsic factor complex occurs in such a small quantity that it can almost be described as a trace substance in the food, and furthermore it is absorbed by an active process (Bockus; Booth).

It is thus possible that the complex requires a long duration of contact with the surface of the intestinal mucosa in order to be absorbed,

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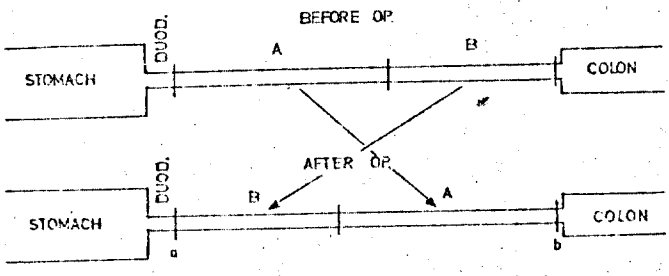

Fig. 1. Schematic diagram of the surgical procedure in transposition of the jejunum and ileum. (*a*) proximal anastomosis; (*b*) distal anastomosis.

and that it is this and not the presence of specific receptors that determines where the absorption of B_{12} takes place. Because of the prevailing motility conditions in the small intestine these prerequisites cannot be fulfilled except in the ileum. In order to shed light on this question with a new experimental approach, the positions of the jejunum and ileum were interchanged, the rat being chosen as the experimental animal. This animal offers advantages over others and has an intrinsic factor mechanism which is similar to that in man (Taylor, Mallet, Wilts & Taylor, 1958; Watson & Florey, 1955).

MATERIAL AND METHODS

Male rats from the same strain (Sprague-Dawley) were used. They weighed about 250 g at the start of the experiments. The material was divided into three groups as defined below. They were fed on a standard diet (Ewos). Before the respective operations the animals were fasted for 18 hours but were allowed free access to water.

Group I: Animals with intestinal transposition

This group consisted of 12 animals, which comprised the first to live more than 2 weeks after the operation. The animals were anaesthetized with ether on an open mask. The small intestine was divided in three places, 1 cm distal to the ligament of Treitz, about 14–16 vessel arcades proximal to and 1 cm proximal to Bauhin's valve. The two segments of small intestine obtained were then interchanged such that the original distal segment (ileum) was placed proximally and the original proximal segment (jejunum) distally (Fig. 1). The respective intestinal segments thus retained their vascular connections. Their original peristaltic orientation was maintained. The intestinal anastomoses were sewn end-to-end with 5–0 silk sutures. Before the abdomen was closed, 10 ml of 0.9% NaCl was deposited in the abdominal cavity. On the first day postoperatively the animals were given 10 ml of the same physiological solution subcutaneously and from the second day they were allowed free access to food and water.

Group II: Sham-treated animals

This group consisted of 8 animals, which were subjected to laparotomy alone, with no interference with the intestine, and given the same pre- and postoperative treatment. These animals were used for study of the body weight course.

Group III: Untreated control animals

This group consisted of 12 animals. They were placed in separate cages in the same way as the operatively treated animals.

The vitamin B_{12} absorption was studied in the animals of groups I and III. At the time of the B_{12} analysis blood was taken from the tail for hemoglobin (Hb) and haematocrit (Hct) determinations. Hb was recorded as cyanmethaemoglobin. Hct was determined by the standard method.

Vitamin B_{12} absorption studies: ^{60}Co (Philips Duphar) with a specific activity of 162 mCi/mg B_{12} was used. The solution was diluted and non-radioactive vitamin B_{12} was added so as to bring the radioactivity to 0.02 μCi/ml and the B_{12} content to 0.003 μg/ml. The solution was then divided into suitable portions and was stored at −20° until required. After completion of the experiment described below the solution was tested for biologically active B_{12} on *Euglena gracilis*. The solution thus contained 2 520 pgB_{12}/ml. The animals were not fed. They were anaesthetized lightly with ether and 1 ml of this solution was deposited in the stomach through a polyethylene catheter. After the administration of radioactive B_{12} the animals were placed in cages where faeces could be collected without admixture of urine. The faeces were transferred daily, for 4 days, to test tubes and analysed for radioactivity. As a standard, 1 ml of the B_{12} solution deposited in plastic tubes in exactly the same way as the solution was administered to the stomach, was used. The samples were then analysed in an Autowell analyser (crystal size 2 1/4″ × 2 1/4″, well 1 1/8″ × 1 3/4″).

The confidence interval for the difference between the "true" values was calculated according to the formula

$$\bar{X}_t - \bar{X}_c \pm 2.6\sqrt{\frac{S_t^2}{n_t} + \frac{S_c^2}{n_c}}$$

where *t* indicates operatively treated animals and *c* controls.

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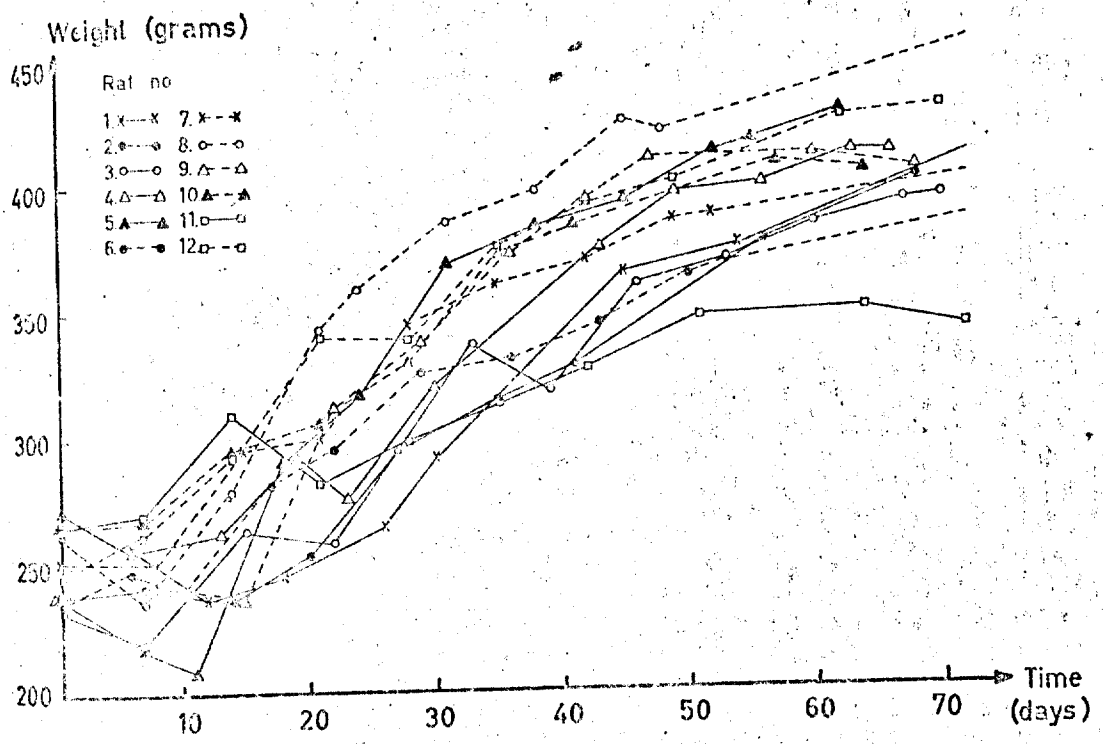

Fig. 2. Postoperative increase in body weight in 12 animals with jejuno-ileal transposition (Group I).

RESULTS

Fig. 2 gives the body weight course in Group I (treated animals). The mean weight at the time of operation was 250 g ($S = 13.6$). Fig. 3 gives the corresponding course in Group II (sham-treated animals) with a weight at operation of 241 g ($S = 4.7$).

The weight curves show the same tendency apart from the immediately postoperative course, where the animals in Group I show a distinct decrease. In order to analyse the weight course more closely, the percentual increase during 60 days was calculated. This is given for Group I in Fig. 4. The mean weight increase was 59% ($S = 12.5$). The percentual increase in Group II is given in Fig. 5. These animals increased their weight by an average of 61% ($S = 10.7$). There is a marked similarity in the weight courses of the different groups, with the exception of the immediately postoperative period.

The values for haemoglobin concentration and haematocrit are presented in Table I. In Group I the mean Hb value was 14.8 g% ($S = 1.1$) and in Group III 14.9 g% ($S = 0.6$). The mean values for the Hct recordings were 46% ($S = 2.8$) and 47% ($S = 2.0$), respectively, in these two groups. No difference between the treated and control animals with respect to these parameters was found.

The results of the vitamin B_{12} absorption studies are presented in Figs. 6 and 7. In Group I the measurements were performed from about 7 weeks to 4 months after the transposition of the small intestine. In making the calculations it was assumed that the administered dose of B_{12} minus the quantity excreted was equal to the quantity absorbed. In Group I there were two deviating results. One animal had an obstruction at the distal anastomosis, a mechanical ileus. The B_{12} absorption was only 20.8% of the dose. The other animal had diarrhoea during the absorption studies, with liquid faeces, and the collection of faeces was difficult and probably not total. No morphological explanation for this was found

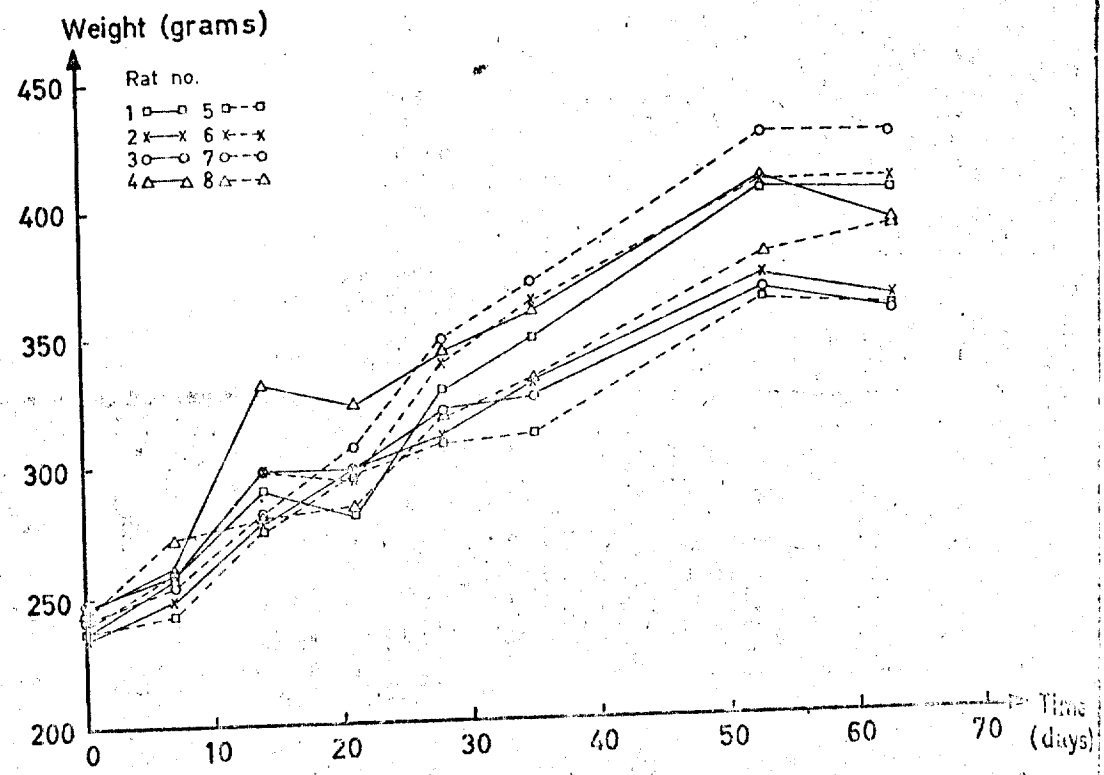

Fig. 3. Postoperative increase in body weight in 8 sham-treated animals (Group II).

macroscopically at autopsy. The absorption was 49%.

There thus remained in Group I 10 animals which during the absorption studies showed a normal faecal consistency and which at autop showed a macroscopically normal small intestin The vitamin B_{12} absorption in these 10 treated animals was 64.8% ($S = 3.3$). In the 12 anima

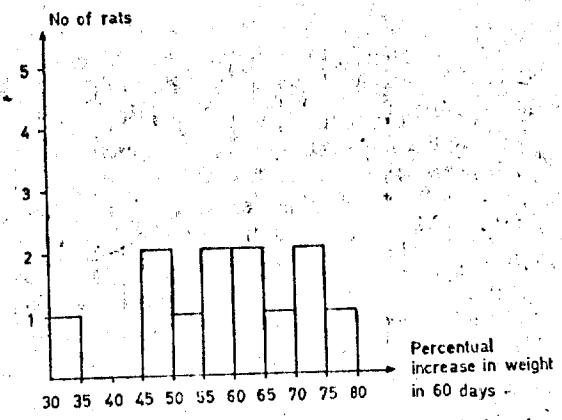

Fig. 4. The percentual increase in body weight during the first 60 days postoperatively in 12 animals with jejuno-ileal transposition (Group I).

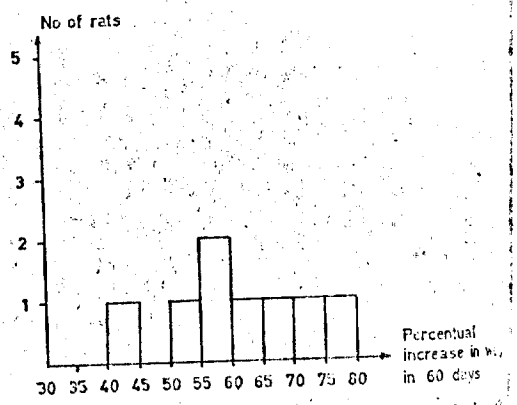

Fig. 5. The percentual increase in body weight during the first 60 days postoperatively in 8 sham-treated ani (Group II).

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Table 1. *Hb and Hct values in animals with jejuno-ileal transposition (Group I) and control animals (Group III) determined in connection with the vitamin B_{12} studies*

Hb, g%		Hct, %	
Treated animals	Normal animals	Treated animals	Normal animals
16.0	14.7	49	47
13.8	14.3	43	45
14.5	14.7	47	47
15.0	15.6	49	47
16.1	14.3	49	43
15.1	15.5	46	49
13.5	14.5	42	49
14.0	16.1	45	50
16.2	14.9	50	46
15.7	15.1	45	48
13.1	14.0	43	45
14.4	15.1	43	48
M14.8	M14.9	M46	M47
S 1.1	S 0.6	S 2.8	S 2.0

of the control group the corresponding mean value was 65.2% ($S = 3.2$).

For the absorption values obtained, the 99% confidence interval for the difference between the "true" mean absorption in Groups I and III, respectively, was −3.2% to +4.0%.

We considered a difference of 4% to be negligible and concluded that with regard to the "true" mean absorption capacity there was no difference between the treated and control animals.

DISCUSSION

The operative procedure on the rat intestine comprised transposition of the distal half of the small intestine (the ileum) to a proximal position, and this intestinal segment now received the food from the duodenum. The jejunum thus became a distal continuation of the ileum.

If the thus reconstructed small intestine retains the propulsive properties of the intact intestine, with rapid propagation through the proximal portion (now the ileum) and a slower passage with a long duration of exposure of the food to the mucosa distally, possible differences between the absorption capacity of these two parts of the intestine should be revealed.

In some of the animals subjected to intestinal

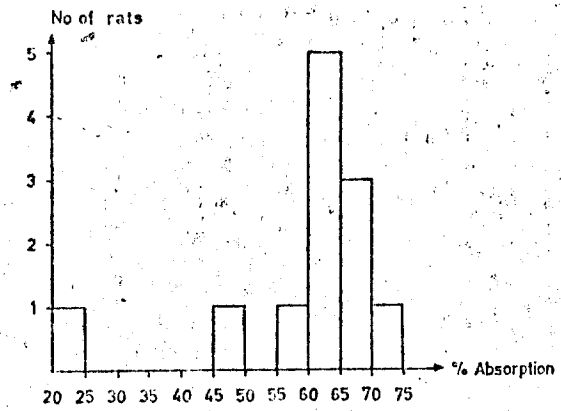

Fig. 6. The percentual absorption of vitamin B_{12} in 12 animals with jejuno-ileal transposition (Group I).

transposition the postoperative body weight decreased during the first 14 days, in contrast to the increase in the sham-treated animals. This difference was assumed to be due to the large degree of trauma which the intestinal operation represented. The difference in operation time and blood loss was considerable. The decrease in weight thus seemed to be an expression of a protracted catabolic stage. When the treated animals had passed this stage the nutrition became adequate and the weight course became identical to that of the sham-treated animals.

The agreement between Groups I and III

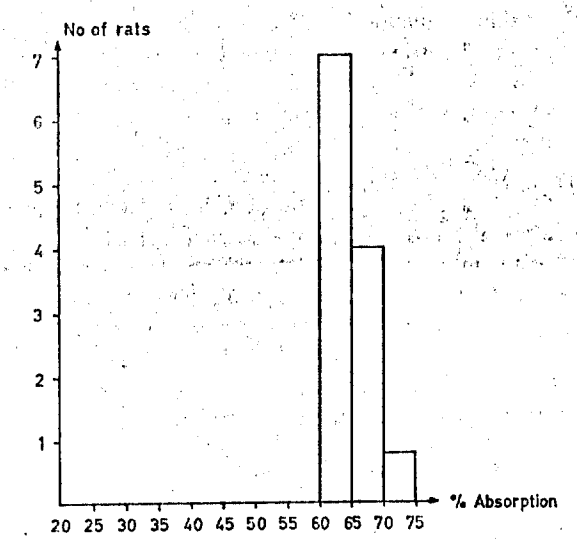

Fig. 7. The percentual absorption of vitamin B_{12} in 12 control animals (Group III).

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concerning the haemoglobin concentration and haematocrit indicates, further, that the transposed small intestine functions satisfactorily with regard to the iron and amino acid absorption that are required for haemoglobin synthesis.

Vitamin B_{12} absorption studies have been carried out on several occasions previously in the rat, and the discussion on where in the small intestine this absorption takes place has been extensive and partly controversial. Booth, Chanarin, Andersson & Mollin (1957) measured radioactively the vitamin B_{12} in the intestinal wall after oral administration and found the largest amount in the middle segment of the small intestine. Using a similar technique, Reynell, Spray & Taylor (1957) found the greatest uptake in the second and third fourths of the small intestine. It was considered that the small uptake in the first fourth might be due to the rapid propagation there. Strauss & Wilson (1958) used the "everted sac" technique in their study. The stimulation by the intrinsic factor, of the vitamin B_{12} uptake in the intestinal wall, was most marked in the middle parts of the small intestine. Okuda (1960) used both oral administration and the "loop technique" to study B_{12} absorption. He found that orally administered vitamin B_{12} passed through the different segments of the gastrointestinal tract at different rates. He considered that the absorption was possibly dependent upon the duration of contact of the food with the intestinal mucosa rather than upon local factors in the intestinal wall. Okuda stated, however, that the middle third of the intestine seemed to be the most important site of B_{12} absorption.

Nygaard (1966) used a resection technique and found that the B_{12} absorption did not constitute a specific property of any particular part of the small intestine, but that the middle segment showed the greatest absorption of this vitamin. Smith & Ellis resected different parts of the small intestine of the rat and conducted experiments with oral administration of vitamin B_{12}. The entire small intestine absorbed B_{12}. Only with excessive resections resulting in diarrhoea did the absorption capacity decrease.

The opinions concerning the location of vitamin B_{12} absorption are thus divided, ranging from the view of Booth (1965) that this absorption can only take place in the ileum and not proximally thereto, through that of Bockus that the surface of the ileal mucosa has specific receptors for B_{12} absorption, to the opinion of Smith and Ellis that the entire small intestine is capable of absorption of this vitamin. The results of the present investigation may possibly deviate from those of previous studies on the rat, where the absorption has been found to vary between 50 and 60%. The standard deviations were greater, however, than in the present investigation. Taylor et al. found the absorption of vitamin B_{12} in man to be about 68%. No reliable explanation for the difference between the absorption value obtained in the present investigation and in previous studies can be given. There are some differences in the method, however. In previous studies ^{56}Co, ^{58}Co or ^{60}Co were used, while ^{57}Co was used in the present study. Mollin (1963) writes the following: "^{57}Co has an unique scintillation spectrum which makes it very useful with well-type scintillation counters." This isotope showed a higher specific activity than those used in previous studies.

The absorption studies thus showed that despite the total jejuno-ileal interchange the vitamin B_{12} absorption took place to the same extent as in the intact control animals. With regard to the propulsive motility after interchange of the proximal and distal segments of the small intestine the following two alternatives may apply: (1) The transposed intestine may acquire the motility pattern of the intact intestine, namely rapid propagation in the proximal part and retardation in the distal part, or (2) A change may take place in the well known propulsive pattern.

Assuming that alternative (1) occurs, the results may support the theory that it is the duration of contact of the B_{12} intrinsic factor complex with the intestinal mucosa that is decisive for the location of B_{12} absorption, rather than the specific and local receptor conditions. Further investigations are needed, however. First and foremost the motility conditions in the transposed segment of the intestine must be determined. The author is proceeding with such studies at the present time.

ACKNOWLEDGEMENT

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ON THE SERUM ACTIVITY OF LACTIC ACID DEHYDROGENASE AND PHOSPHOHEXOSE ISOMERASE IN PERNICIOUS AND HEMOLYTIC ANEMIAS

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Lactic acid dehydrogenase (LDH) occurs in varying concentrations in the different cellular systems of the body. The main source of LDH normally occurring in serum is not yet known. Wieland & Pfleiderer (1957) have shown that LDH from different organs is not represented by identical proteins.

Increased serum LDH[1] activity has been observed in a number of diseases, including myocardial infarction (Wróblewski 1957, Hsick & Blumenthal 1956), different types of leukemia (Wróblewski 1957, Zimmerman & Weinstein 1956, Hsick & Blumenthal 1956), muscular trauma (Wróblewski 1957), liver diseases (Wróblewski 1957, Zimmerman & Weinstein 1956, Hsick & Blumenthal 1956), renal diseases (West & Zimmerman 1958), different types of anemia (Wróblewski 1957, Hess & Gehm 1955, Lührs & Negelein 1956, Zimmerman & Weinstein 1956, Zimmerman, West & Heller 1958, Gordin & Enari 1959, Cintrón-Rivera, Acosta-Matienzo & Diaz-Rivera 1959, Heller, West & Zimmerman 1959, Levitan, Wasserman & Wróblewski 1959), general carcinomatosis (Wróblewski 1957, Lührs & Negelein 1956, Zimmerman & Weinstein 1956, West & Zimmerman 1958, Hsick & Blumenthal 1956). Exceptionally high values have sometimes been noted in vitamin B_{12} deficiency, but it appears that no attempts have been made to find an explanation of this phenomenon.

In 16 cases of pernicious anemia (p.a.) Hess and Gehm (1955) found serum values 5—21 times normal. Lührs and Negelein (1956) describe two patients with p.a., in whom the LDH was 8 times the normal values. In 12 cases of megaloblastic anemia, including 7 with p.a., Zimmerman *et al.* (1958) found LDH to be only 2—3 times the upper normal limit. Gordon and Enari (1959), on the other hand, observed markedly raised values in p.a. and pernicious tapeworm anemia. During vitamin B_{12} therapy the values rapidly returned to normal.

The red blood cells contain LDH in a

[1] Hereafter the abbreviation LDH will be used to denote the recorded activity of lactic acid dehydrogenase.

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concentration about 220 times that in the serum, but in hemolytic anemia, both acquired and congenital, only slightly raised or normal values have been found in the serum (Zimmerman & Weinstein 1956, Zimmerman *et al.* 1958).

Phospho hexose isomerase (PHI), which catalyses the conversion of fructose-6-phosphate to glucose-6-phosphate and *vice versa*, also occurs in the serum with abnormally high activity[1] in a number of pathologic conditions, including acute hepatitis (Bruns & Hinsberg 1954), leukemia (Blanchaer, Green, Maclean & Hollenberg 1958), cancer (Bodansky 1954, Bruns & Jacob 1954), diabetes mellitus (Bruns & Jacob 1954) and megaloblastic anemia (Heller, West & Zimmerman 1959).

In the present investigation the LDH in the serum from patients with p.a. was compared with that in hemolytic anemia. In some of the patients the serum PHI was also determined.

In 4 patients with p.a. the activities of these enzymes in the plasma were studied during remission after B_{12} therapy.

MATERIAL

The material consisted of sera from 27 cases of p.a. and 16 cases of hemolytic anemia.

The diagnosis p.a. was based on the demonstration of megaloblastic bone marrow in all cases except 2, where typical megaloblasts were found, but where the concentration of vitamin B_{12} in the serum was subnormal (see Table II). The correctness of the diagnosis was confirmed farther by the presence of histamine refractory achylia, and in some cases by the result of the Schilling test and by remission during the course of B_{12} therapy.

In 10 cases with p.a. the LDH was determined in the serum as well as in the red blood cells.

The hemolytic anemias consisted of 6 cases of congenital hemolytic anemia, 3 cases of hemolytic anemia caused by phenacetin and 7 cases of acquired hemolytic anemia of unknown origin.

The normal material for determination of LDH and PHI in the serum consisted of sera from 53 and 54 blood donors respectively. Whole blood from 29 apparently healthy persons was used as normal material for determination of LDH in the red blood cells.

METHODS

LDH in the serum and the red blood cells. — The LDH was determined according to Wroblewski and La Due (1955). The LDH in the red blood cells was calculated on the basis of the hematocrit and LDH values of serum and whole blood. In the last mentioned determinations the red blood cells were hemolysed by addition of water.

PHI in the serum. — The PHI was determined according to Bodanski (1954). The activity is expressed in units differing from Bodanski units. On determination according to Bodanski the amount of fruktose-6-phosphate (F-6-P) formed is determined by Roe's fructose determination method and before every determination calibration is done with standard solutions of F-6-P. To simplify the method, fructose was used as standard in the present investigation. One unit is to be understood as the F-6-P formed, which gives the same extinction on determination of fructose according to Roe, as 1 microgram of fructose.

RESULTS

It is clear from Table I that in pernicious anemia as well as in hemolytic anemia the LDH and PHI in the serum are increased. The relative deviation from the normal values was greatest for LDH in both conditions. Fig. 1 gives the serum LDH in relation to the hemoglobin content of the blood in the

[1] Hereafter the abbreviation PHI will be used to denote the activity of phosphohexose isomerase.

Table I.

	Number	Serum LDH in U./ml./min.		Serum PHI in U.	
		Mean	Range	Mean	Range
Normals*	53	266	110—422	—	—
Pernicious anemia	27	5360	300—15900	—	—
Hemolytic anemia	15	575	140—1770	—	—
Normals*	54	—	—	7.0	2.1—11.9
Pernicious anemia	14	—	—	30.3	11.3—45.2
Hemolytic anemia	9	—	—	8.9	5.6—16.7

*For the normals the range is given as the mean ± 2 standard deviations.

individual cases. The activity was higher when the concentration of hemoglobin was low than when it was high, and at the same concentration of hemoglobin it was higher in cases of p.a. than in cases of hemolytic anemia. The same tendency was found for PHI (Fig. 2).

Values for the 2 cases of p.a. without

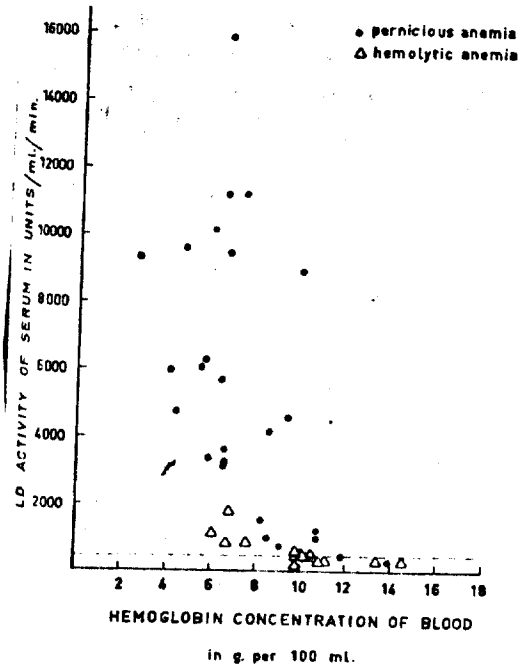

Fig. 1. The serum LDH in relation to the hemoglobin content of the blood. The line marks the upper normal limit of serum LDH.

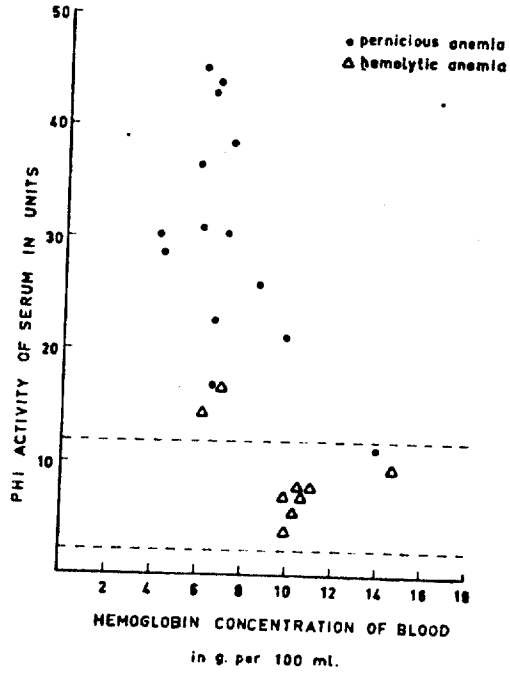

Fig. 2. The serum PHI in relation to the hemoglobin content of the blood. The lines mark the normal limits of serum PHI.

Table II.

B_{12} in serum Picograms/ml.	LDH in serum U./ml./min.	PHI in serum U.
25	1000	—
25	300	11.3

The values of the 2 cases of p.a. without megaloblasts in the sternal puncture.

megaloblasts in the sternal puncture are given in Table II.

In 4 cases of p.a. the serum LDH and PHI were followed during treatment with vitamin B_{12}. A temporary rise (see Figs. 3 and 4) was sometimes noted, after which the activity for both enzymes decreased rapidly.

The LDH was determined in 10 cases of p.a. also in the red blood cells. The mean value was 67 700 and the lowest and highest values were 31 000 and 100 000 U./ml./min. respectively. For the normal material the mean was 76 000 and the normal range (mean ± 2 standard deviations) 60 000—92 000 U./ml./min. The difference between the means was not significant, but the range of variation was clearly wider in p.a. than in the normals.

DISCUSSION

The serum concentration of an enzyme depends on the ratio between the inflow and the elimination of the enzyme, but when the concentration of the enzyme is determined indirectly by measurement of the activity, the value obtained is influenced by the presence of any activators or inhibitors. Substances in

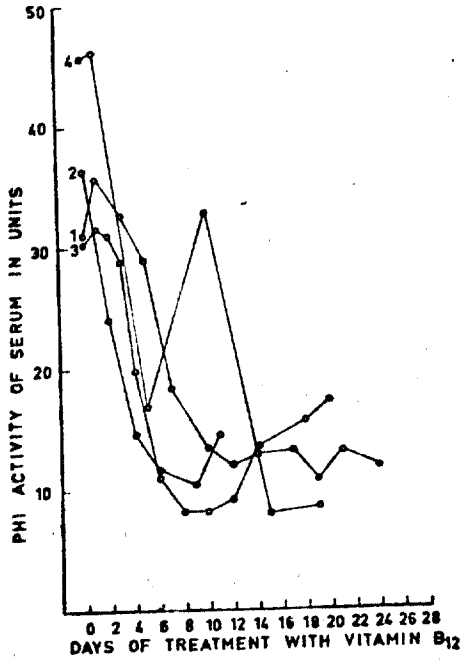

Fig. 3. LDH in serum during treatment of pernicious anemia with vitamin B_{12}.

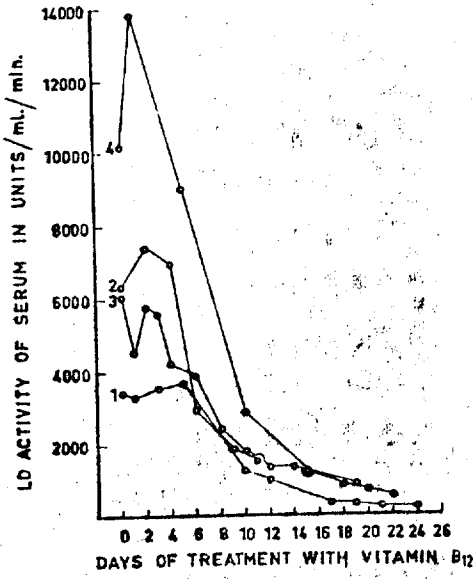

Fig. 4. PHI in serum during treatment of pernicious anemia with vitamin B_{12}.

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serum from healthy persons and from patients, which inhibit or activate LDH and PHI have not been studied in further detail. It is generally believed that the activity of these enzymes in the serum largely reflects the concentrations.

It is clear from the results that the increase of LDH was excessive in advanced cases of p.a., in contrast to what is found in advanced hemolytic anemia, where the serum values are admittedly very pathologic, but of the same order as in myeloid anemia and advanced liver disease, for example. The PHI values showed the same trend but the changes were less pronounced.

In anemia, serum values exceeding 3000 U./ml./min. for LDH could be regarded as pathognomic of vitamin B_{12} deficiency, but they are of less diagnostic value because the early cases with only a slight lowering of the number of red blood cells in the circulation show moderate or no LDH increase in the serum.

The LDH content in circulating red blood cells in patients with p. a. is of the same order as in normals. The markedly increased inflow of LDH into the blood stream in p.a. may be due to increased rate of destruction of red blood cells. In p.a. a slightly shortened survival time of the red cells can be demonstrated with Cr-tagging of the red blood cells, but the plasma iron turnover, the stercobilin excretion and the endogenous CO-formation show that the total hemoglobin turnover must be much greater than what is obtained on calculation according to Cr-tagging. A considerable so-called bone marrow hemolysis is probable. In the normal metabolism of aged red cells as in most hemolytic anemias, on the other hand, the main destruction of the blood cells occurs extravascularly (Jandle, Jones & Castle 1957; Noyes, Bothwell & Finch 1959).

If the rate of elimination of the LDH molecule from the blood stream, the LDH in the serum and the LDH concentration in the red blood cells are known, it is possible, according to the following formula, to calculate how large the daily intravascular hemolysis must be for maintenance of the LDH activity found in the serum if it is assumed that all serum LDH emanates from erythrocytes and that the distribution volume of LDH is equal to double the serum volume.

Formula

per cent hemolysis of circulating blood cell mass

$$\frac{2 \cdot \frac{0.693}{T/2} \cdot (1\text{-Hkr}) \cdot LDH_{serum} \cdot 100}{Hkr \cdot LDH_{erythrocytes}}$$

where T/2 = half time of serum LDH in days

Hkr = hematocrit

A maximum value for the half time of serum LDH was calculated graphically for 4 cases of p.a. during treatment with vitamin B_{12}. Only the LDH values recorded 4 days or more after commencement of therapy were included. The pathologic increase of the LDH inflow in, or the pathological decrease of the LDH elimination of the serum then appeared to have stopped. The fall in the LDH values in relation to the time agree then with the elimination according to a simple exponential course. The value found for the half time, 3 days, gives approximately the highest possible true value.

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Table III.

LDH, U./ml./min.		Per cent daily hemolysis of circulating blood cells for maintenance LDH in serum	Calculated survival time in days
in serum	in erythrocytes		
440	31000	0.20	500
650	42000	0.17	588
1150	75000	4.09	24
790	72000	1.26	79
1250	70000	2.08	48
1000	97000	1:52	66
11500	100000	31.16	3.2
5000	81000	10.66	9.3
490	46000	0.87	115
510	59000	1.13	88

The steady state of LDH in the plasma of normals corresponds to a daily hemolysis of the circulating red blood cell mass of 0.36 per cent, which corresponds to an average erythrocyte survival time of 277 days, if the serum LDH emanates mainly from destroyed red blood cells — an improbable assumption. The survival time is normally about 120 days, which corresponds to a daily hemolysis of 0.83 per cent. From the value obtained it can be concluded that the bulk of LDH in the blood cells that undergoes normal, extravascular hemolysis is not returned in active form to plasma.

Corresponding calculations were made in 10 cases of p.a. where LDH was determined in the serum and red blood cells and where the hematocrit value was known. Results are given in Table III. In the 8 cases of p.a. with the highest serum LDH according to Fig. 1, similar calculations were made. The hematocrit and LDH in the erythrocytes were not determined in these cases. Therefore, the normal value for LDH in the erythrocytes, 76 000 U./ml./min., was taken. The hematocrit values were calculated from the number of erythrocytes per mm^3 on the assumption that the mean volume of the megalocytes was 1.3 times that of normocytes. Calculations showed that in these 8 cases of p.a., maintenance of the serum LDH would require a daily *intravascular* hemolysis of 16—18 per cent of the circulating erythrocytes on the assumption that LDH emanated solely from the red blood cells. Values of the same order are also given in Table III. They correspond to a mean survival time of the erythrocytes of 6.2—0.15 days and are thus unreasonable. This shows that the increased activity of LDH in the serum in p.a. emanates not only from the hemolysed circulating red blood cells. The molecular weight of the LDH is probably more than 100 000, so a more or less general cell leakage is improbable. The results suggest that the very high LDH values in serum in advanced B_{12} deficiency reflect severely disturbed cellular function, probably with shortened cell survival in the

bone marrow (*e. g.* megaloblasts) or in other tissues.

The pathways by which the LDH and PHI are eliminated are not known, nor is the elimination mechanism. Evidence from animal experiments (West & Zimmerman 1958) suggests that the LDH is normally not excreted in the urine. It cannot be excluded *à priori* from the present investigation that B_{12} directly or indirectly affects the elimination rate and that the high level of LDH in the serum in a steady state in B_{12} deficiency may be explained by a slower elimination.

SUMMARY

Serum activity of lactic acid dehydrogenase (LDH) and phosphohexose isomerase (PHI) were studied in cases of hemolytic anemia and pernicious anemia. The latter showed very high relative increase in activity of both enzymes compared with normal. Values of LDH exceeding 3000 U./ml./min. in anemia argue strongly for pernicious anemia. The half time of LDH in serum was determined approximately in cases of pernicious anemia during treatment with vitamin B_{12}. On the basis of the results the possible cause of increased serum LDH are discussed.

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Effect of Vitamin B_{12} on Experimental Hepatic Injury.* (17686)

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Observations on the effect of crude liver extract in experimental hepatic injury in rats have been previously reported(1-3). It has been shown that liver extract given by mouth is without influence on, or will even promote the development of acute diffuse hepatic necrosis. On the other hand, liver extract will not only prevent cirrhosis but has a definitely beneficial effect in the treatment of hepatic cirrhosis. Thus, the effect of liver extract may be comparable to that of choline, the latter also having a beneficial effect on cirrhosis, and no effect or a deleterious one on necrosis(1,4,5). The fact that the combination of liver extract with methionine or with protein proved to be superior in the treatment of experimental cirrhosis(3) to the effect of methionine or protein alone seemed to indicate that the beneficial effect of the liver extract might not have been due to its content of choline but perhaps to some other constituent, such as vitamin B_{12}. This supposition was based on the assumption that choline, methionine and protein (caesin) all act similarly as sources of "labile methyl" and it was difficult to believe that a simple increase in supply of the same factor over and above a sufficient supply could have improved the therapeutic results as distinctly as was seen on the addition of liver extract to methionine and protein.

In the course of their studies on the lipotropic effect of liver extract Hall and Drill(6) reached similar conclusions. They found liver extract a much more potent lipotropic agent

* This represents work carried out under the auspices of the Commission on Liver Disease of the Armed Forces Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D.C.

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than could have been expected from its content of choline alone. A more direct proof of the sparing effect of vitamin B_{12} on the choline ("labile methyl") requirement of rats and chicks was first presented by Schaefer, Salmon and Strength(7). They were able to prevent "hemorrhagic kidney" in rats, and promote growth in chicks with suboptimal amounts of choline and methionine, provided B_{12} was added to the diet (3 γ/100 g). This interrelationship between vitamin B_{12} and methylating compounds has been confirmed on chicks by Gillis and Norris(8).

In the light of this new evidence it became desirable to study the effect of B_{12} as such in various forms of liver injury, in order to be able to distinguish between the effects of choline and B_{12} in liver extract.

Massive hepatic necrosis. Hepatic necrosis in rats fed a diet low in casein occurs but only very irregularly(1,9). A more severe, truly massive hemorrhagic necrosis of the liver can be produced in almost 100% of the experimental animals by using a diet with a proper preparation of yeast as sole source of protein(5,10-12). Yeast is free from B_{12}(13), whereas casein, if not especially purified, may contain traces of B_{12}. Thus the question arises whether a deficiency of B_{12} may play a part in the development of this especially severe fulminating massive hepatic necrosis when yeast rations are used. It may even be surmised that the irregular occurrences of hepatic necrosis in rats fed casein-containing rations are related to traces of B_{12} remaining in the purified casein. The often observed, slightly injurious effect of liver extract in this form of hepatic necrosis would then be due to the presence of choline.

This assumption, however, was not borne out by experiments in which the incidence of hepatic necrosis was compared in groups of rats receiving a "necrogenic" yeast ration with or without supplements of vitamin B_{12}. The composition of the yeast rations was as follows: A. Yeast,† 18; corn starch, 79; salt mixture (USP II), 3. B. Yeast corn starch, 59; lactose, 20; salt mixture (USP II), 3. Peanut oil, 0.5 ml and cod liver oil, 2 drops, were added for each 8 g of the dry mixture (ration A or B). All animals received daily 20 γ "Synkayvite"‡ and supplements of 20 γ thiamine, 25 γ riboflavin, 20 γ pyridoxine, and 100 γ calcium pantothenate, dissolved together in 1 ml water. Rats were of the Sprague Dawley strain, males, weanlings, with an average weight of 50 g. Twelve rats received ration A without supplement of B_{12}§, 6 rats received the same ration with vitamin B_{12}, 1 γ daily by mouth. Seven additional rats were fed ration B with vitamin B_{12}, 1 γ daily by mouth. Thus, altogether 13 rats received a necrogenic yeast diet with supplement of vitamin B_{12}, and 12 rats were used as controls without B_{12}. All rats in both groups died from typical massive hemorrhagic necrosis, involving preferentially the left lobe or left half of the liver. There was no statistically significant difference in length of survival of the animals in the two groups; rats fed without B_{12} died on the average after 34, those receiving supplement of B_{12} after 36, experimental days. Thus, vitamin B_{12} given by mouth was without any influence on the production of massive hepatic necrosis in rats receiving a "necrogenic" yeast diet.

Lipotropic effect of B_{12}. Experimental dietary hepatic cirrhosis is due to insufficient supply of lipotropic factors, and is accompanied, at least initially, by fat infiltration of the

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† "D.C.L. Vit. B_1 yeast" from Distillers Co., Ltd., Glasgow C.4, Scotland.

‡ Water-soluble vitamin K preparation, Hoffmann-LaRoche, Nutley, N.J.

§ Kindly supplied by Chas. Pfizer and Co., Inc., Brooklyn, N.Y.

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liver. Thus, it may be assumed that substances with lipotropic activity should have a beneficial effect on the production and perhaps even on the treatment of dietary hepatic cirrhosis. Direct evidence may only be obtained in long-term experiments in which the effect of the substance in question is studied on the prevention and treatment of hepatic cirrhosis. Such investigations on the effect of B_{12} in the treatment of dietary cirrhosis of the liver are in progress.‖ The present report includes experiments designed for the study of the lipotropic activity of B_{12}.

The same general procedures were followed as were previously reported(14,15). Young adult female rats of the Sprague Dawley strain, weighing 145-175 g, were used. As usual, control and treated animals were run simultaneously. When all of the animals of an experiment could not be run at one time, equal numbers of the various groups were started together. Two types of rations were used: a) Low protein-high fat (V_4), and b) Low protein-low fat (V_{11}). V_4 *(Exp. 31)*: Casein, G.B.I. Vitamin Test, 8; "Vream"¶, 40; Sucrose, 48; Salt mixture (USP II), 4. V_{11} *(Exp. 33:* Casein, 8; "Vream", 5; Sucrose, 83; Salt mixture, 4. Each animal received daily 20 γ thiamine, 25 γ riboflavin, 20 γ pyridoxine, 100 γ calcium pantothenate dissolved in 1 ml of water. Three drops of percomorph oil and 3 mg of α-tocopherol were given weekly. When methionine was given, the daily dose (50 mg dl-methionine)** was dissolved in 1 ml of water and mixed with the vitamin B supplement. B_{12} was administered similarly, in form of a solution contain-

TABLE I.

The Lipotropic Effect of B_{12}. Ten rats in each experiment.

Exp.	Diet	Treatment	Liver Wt, g	Liver Total fat, %	Rat weight Initial, g	Rat weight Change, %	Food intake, g/day
31	V_4 (High Fat)	Control	7.7 ± .40	27.8 ± 2.6	156	− 8.8 ± 3.3	6.1 ± .36
		B_{12}	6.7 ± .24	23.0 ± 2.2	156	− 8.7 ± 1.2	5.9 ± .19
		Methionine	6.5 ± .41	18.3 ± 1.4	152	− 7.0 ± 1.6	5.5 ± .39
		Meth. + B_{12}	5.9 ± .35	14.7 ± 1.6	152	−11.3 ± 1.8	5.0 ± .25
33	V_{11} (Low Fat)	Control	9.3 ± .44	35.0 ± 1.7	160	−12.7 ± 2.1	8.1 ± .36
		B_{12}	8.0 ± .56	20.5 ± 1.6	158	− 2.6 ± 1.4	9.4 ± .20
		Methionine	6.5 ± .48	14.6 ± 1.8	161	− 8.3 ± 2.7	8.1 ± .43
		Meth. + B_{12}	6.6 ± .27	10.4 ± 1.3	158	− 4.5 ± 2.9	8.6 ± .41

‖ Investigations are also carried out at the Army Hepatic and Metabolic Center, Valley Forge General Hospital, on the metabolic and therapeutic effect of B_{12} in man (normals and patients with subacute hepatitis and cirrhosis), in collaboration with Dr. V. M. Sborov and Lt. D. Sutherland.

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¶ Vegetable shortening, obtained from Swift & Co., Chicago.

** Kindly furnished by Wyeth, Inc.

ing 0.5 γ of the crystalline compound.†† At the end of the 21 day experimental period, the animals were sacrificed and the liver analyzed for total lipide with the same technique used in the previous experiments.

The results obtained are summarized in Table I. In Exp. 31, using a low protein-high fat diet, administration of B_{12} was followed by a slight statistically insignificant reduction of the relative liver fat content. When combined with methionine, B_{12} had increased only slightly, without statistical significance the lipotropic effect of methionine alone. It should, however, be pointed out that the average weight of the liver, and in consequence, the absolute fat content of the liver in Exp. 31 were distinctly reduced in the groups of animals receiving supplements of B_{12} (with or without methionine) to their low protein-high fat ration.

The lipotropic effect of B_{12}, suggested in Exp. 31, became clearly evident in Exp. 33 when the low protein-low fat diet was used. It appeared to be especially marked in the group of animals receiving B_{12}, without methionine, as supplement to the basal ration. The slightly increased average food intake and better average weight curve in the groups of animals receiving B_{12} over those in the control groups should have, if anything, counteracted to some extent the lipotropic effect of B_{12}(16,17).

The lipotropic effect of a B_{12} concentrate was found recently also by Drill and McCormick(18). These authors used a lipotropic diet with moderately high protein (16%) and high fat (51%) content. The B_{12} concentrate was given by injection (0.2-1.0 γ, 3 times per week).

†† Kindly supplied by Merck & Co.

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We were able to show that B_{12} may exert its lipotropic effect also when given by mouth. Further, the experiments here reported indicate that a low protein-high fat diet will hinder the demonstration of lipotropic activity by B_{12}, whereas a low protein-low fat diet, or, as in the experiments of Drill and McCormick, a moderately high protein-high fat diet, will not interfere with the lipotropic reaction following the administration of B_{12}. These observations may also be related to the findings of Bosshardt, Paul and Barnes(19) on the growth-promoting effect of B_{12} in mice receiving varying proportions of fat and protein in their diet. Special studies are required to answer the questions whether with increased dosage of B_{12} the lipotropic effect may become evident in rats fed a low protein and high fat diet, or whether the lipotropic effect as such may be potentiated by giving higher amounts of B_{12}. It should be pointed out that in our experiments methionine has proved to be a more effective lipotropic agent than vitamin B_{12}. Experiments are contemplated to show whether additional amounts of vitamin B_{12} will increase its lipotropic effect, or whether the sparing effect of vitamin B_{12} could be made more evident with reduced suboptimal amounts of the lipotropic compound (choline, methionine).

Summary. Vit. B_{12} (1 γ daily, by mouth) had no effect on the development of massive hepatic necrosis in rats fed experimental rations with yeast as sole source of protein.

Vit. B_{12} (0.5 γ daily by mouth) has shown significant lipotropic activity in rats fed a low protein-low fat ration. Under the experimental conditions chosen methionine was more effective than vitamin B_{12} in preventing fat deposition in the liver. A low protein-high fat diet seemed to interfere with the lipotropic effect of B_{12}.

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Effect of Vitamin B_{12} and Cortisone on the Glucose Tolerance of Aged Persons*

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It is known that in advanced age diabètes occurs more frequently. In the course of screenings made in two homes for aged persons, we examined all inmates over the age of 70. Apart from atherosclerosis, the persons submitted to our examinations were considered healthy.

According to *Hoppe-Seyler*[23] the diabetes of old age is caused by atherosclerotic alterations of the pancreatic vessels. This statement is still valid[1]. *Bürger* believes that in senile states a physiologic insufficiency of pancreas develops. As early as 1922 *Löffler*[28] established the existence of diabetic-like blood sugar curves in aged persons. *Kohl* and *Dahmann*[27] studied the glucose tolerance using 1 g/kg glucose *per os* and concluded that with the advance of age this tolerance shows a progressive decrease. According to *Klotzbücher*[26] the Staub-Traugott effect is lost, while the pancreas is incapable of mobilizing insulin. The insulin-resistance is increased after *Stöcker*[35]: the fall of the blood sugar level following insulin injection is less noteworthy than in young persons. *Bürger* explained that this fact is due to the lower glycogen of liver and musculature.

Hoffstätter and co-workers[22] found in 1945 no remarkably high rise in carbohydrate sensitivity, *Horváth* and co-workers[24] noted a slight diminution in tolerance. *Deren*[8] observed prolonged sugar tolerance curves. *Marshall*[31] found many variations in a group of 27 healthy men of an average age of 72. However, the latest literary data are rather concordant. *Simon* and *Garvey*[31] observed that the blood sugar and the sugar metabolism is more labile and disturbed in patients with senile psychosis than in chronic schizophrenic patients. *Rush* and *Tupper*[32] performed the routine 2-hour postprandial test and observed a decreased tolerance for glucose with advancing age; this procedure revealed an incidence of diabetes mellitus of 12.6%, with another 7.5% of the patients classified as possibly having diabetes. *Sandric* and *Ghiotto*[33] as well as *Gottfried* and co-workers[13], noted that the diabetic curves ranged from a minimal to a moderate degree. *Fumagalli* and *Sodi*[9] established that in advanced age the blood insulin level is decreased and the

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insulin response is of a lesser degree. These results would appear to show that the endocrine pancreas undergoes a functional decrease in old age.

Recently, in order to increase the sensitivity of the glucose loading, ACTH, cortisone and especially prednisolone were used[1-12]. Examining the value of this procedure on the relatives of diabetic patients, it became evident that in aged persons the sugar curves appeared to be more pathologic than in the young[37]. Using this method, *Brandt*[2] found a great incidence of latent diabetes among aged persons. *Magyar* and *Márton*[30], administering 30 g of glucose per os, found diabetogenic values in approximately half of the cases.

Methods

The glucose tolerance tests were made with 1 g/kg glucose p.a. dissolved in 250 ml of water in the early morning hours, after 12 h fasting. Blood sugar determinations were made according to Hagedorn-Jensen's method, using 3 parallels, before the ingestion of glucose as well as 30–60–120 minutes after.

Five to seven days later the tests were repeated, partly with vitamin B_{12}, partly with prednisolone. 1000 gamma-vitamin B_{12} was injected subcutaneously after sampling of the fasting blood sugar. In these cases the glucose was ingested 30 minutes after the injection of vitamin B_{12}. (Vitamin-B_{12} injections of "Richter"*, brand "Trifavit" = Vitamin-B_{12} factor III were used.) In some of our experiments two hours before examination 10 mg prednisolone (tablets of delta-1-hydrocortisone "Richter"*) were given.

Results

Table I illustrates the glucose tolerance of 50 aged persons (30 women and 20 men over 70 years).

Table I

	% of Patients	
	Glucose	Prednisolone-glucose
1. Fasting blood sugar level exceeds 120 mg%	4	20
2. Maximum rise of blood sugar above 200 mg%	14	40
3. Blood sugar level 2 hours after ingestion of glucose remains 140 mg%	24	47
4. Compared to the fasting values, the blood sugar level following ingestion of glucose exceeds +90%	44	63
5. 2-hour value is above the fasting value with 40%	36	50
6. Normal tolerance	42	20

The tolerance is considered to be pathologic in those cases where: 1. the fasting blood sugar level exceeds 120 mg%; 2. the maximum rise of blood sugar is above 200 mg%; 3. the blood sugar level two hours after ingestion of glucose remains above 140 mg%;

* We express our appreciation to the Richter Chemical Works of Budapest for the necessary drugs.

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4. compared to the fasting value, the blood sugar level following ingestion of glucose exceeds + 90%, and 5. when the 2-hour value is 40% above the fasting value. When one of the above-mentioned factors is present, then the carbohydrate tolerance may not be considered as altogether normal. Based on the above, from the 50 aged persons 29 indicated a pathological tolerance (= 58%). In 21 cases (= 42%) this proved to be normal. Two (4%) of these may be considered as suffering from diabetes.

In the majority of cases, after sensitization with prednisolone the blood sugar curves became pathologic. If the pathologic curves are judged according to the above-mentioned five factors, then only 6 (= 20%) of the total of 30 examined aged persons should have a normal tolerance, while 80% were pathologic (see table I).

In the majority of cases administration of vitamin B_{12} improved the glucose tolerance and facilitated the storing of glucose in the tissues. Checking statistically – according to *Student* – the differences existing between the 2-hour values, show that the effect of vitamine B_{12} is significant: $t = 2.82$; $p = 0.01$. If the effect of vitamin B_{12} is calculated not only on the basis of the 2-hour values, but together with the 30′ and 60′ values, the effect of vitamin B_{12} upon the improvement of the glucose tolerance is even more considerable: $t = 3.8$; $p = < 0.01$ (fig. 1).

Vitamin B_{12} influenced in a lesser degree the carbohydrate tolerance by the prednisolone-glucose test. The difference between

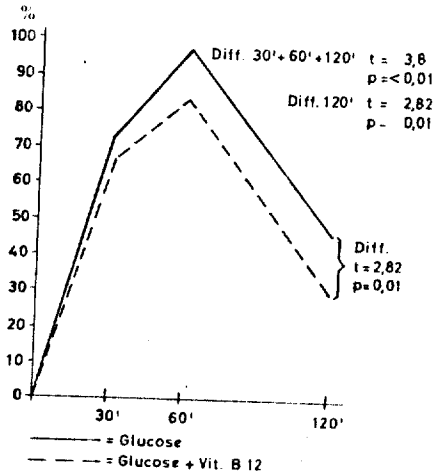

Fig. 1. Glucose tolerance of aged persons.

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the 2-hour values is significant ($t = 2.31$; $p = 0.03$). Thus vitamin B_{12} improves the glucose tolerance even after prednisolone sensitization. If, however, the 30, 60, 120-minute values are calculated, then the effect of vitamin B_{12} is questionable ($t = 1.93$; $p = 0.06$–0.07), as illustrated on table II.

Table II

Modification of Blood Sugar Level

	30	60	120	Diff. 30-60-120	Diff. 120
Prednisolone-glucose	+72.2%	+99.4%	+45.64%	t = 1.93 p = 0.065	t = 2.31 p = 0.03
Prednisolone-glucose + vitamin B_{12}	+71.5%	+89.4%	+31.10%		

Discussion

In experiments made with a screening technique in the homes for aged, we found that in 50 persons over the age of 70 the glucose tolerance indicated a decrease in 58% of the cases and 2 (= 4%) of these suffered from diabetes mellitus. Sensitization with prednisolone results in the upward tendency of the diabetogenic curves. In some cases the blood-sugar curves were pathologic also in the course of the initial glucose loading. Following prednisolone sensitization these curves did not turn more pathological and the only modification noted was related to the shape of the curve. We believe that in these cases the decrease of the glucose tolerance was mainly due to factors other than insulin.

One may question whether aged persons may be considered as latent diabetic cases. It is more correct to consider aged people as being in a pre-diabetic state. Consequently, the diet should be established accordingly. Since the modern diabetes diet corresponds to that applied in atherosclerosis, we maintain that the diet of old people should be adjusted accordingly.

Apart from these considerations, we agree with the opinion of those[30] who hold that the decreased glucose tolerance of old age should not be attributed solely to the lowered insulin secretion, other factors (for example decreased muscle activity etc.) participating as well.

Vitamin B_{12} stimulates the enzymatic activity of tissular phos-

phatases in the intestine[16] and facilitates the absorption of glucose in malabsorption of *M. Addisoni*, *M. Simmonds*, *Sheehan*-syndrome, myxoedema[17, 19].

The deposition of glucose in the tissues is also achieved through phosphorylation. Vitamin B_{12} increased the glucose tolerance in several diseases where this proved to be lowered, for example hyperemesis gravidarum[29], *M. Addison*[11], scarlet fever[15], epidemic hepatitis[18], etc. In diabetes mellitus, however, vitamin B_{12} improved the tolerance only in a slight proportion of cases[21].

According to *Chemnitius*[5], as well as our own studies[20], vitamin B_{12} possesses a certain antagonistic effect towards cortisone. We therefore examined whether administration of 1000 gamma-vitamin B_{12} counteracts the diabetogenic effect of prednisolone. Calculating the 2-hour values, this effect may be termed as statistically significant ($t = 2.31$; $p = 0.03$).

Summary

The carbohydrate metabolism of 50 persons over the age of 70 years, living in homes for old people, was studied. The glucose tolerance was decreased in 58%. In 4% manifest diabetes could be established.

The prednisolone-glucose test was pathologic in 80% of the cases. In most cases vitamin B_{12} (doses of 1000 gamma) increased the tolerance, reducing the 2-hour value of the blood sugar. The diabetogenic effect of prednisolone in aged persons is moderately reduced.

It seems to be correct to consider aged people as being in a prediabetic state and therefore the diet of old age should be adjusted accordingly.

Zusammenfassung

An 50 Personen über 70 Jahren aus Altersheimen wurde der Kohlehydratstoffwechsel untersucht. Die Glucose-Toleranz war in 58% vermindert. In 4% konnte Diabetes festgestellt werden.

Der Prednisolon-Glucose-Test war in 80% der Fälle pathologisch. Vitamin B_{12} (1 mg Dosen) erhöhte die Toleranz indem der 2-Stundenwert des Blutzuckers gesenkt wurde. Die diabetogene Wirkung von Prednisolon ist bei alten Personen etwas vermindert.

Es scheint angezeigt, alte Leute als in einem prädiabetischen Stadium zu betrachten und ihre Diät entsprechend zu wählen.

Résumé

Le métabolisme hydrocarboné de 50 personnes de plus de 70 ans vivant dans des maisons de retraite a été étudié par les auteurs. La tolérance à la surcharge en glucose est diminuée de 58% en moyenne. Un diabète manifeste a été trouvé chez 4% des sujets.

Le test au prednisolone-glucose donne des valeurs pathologiques dans 80% des cas. Une dose de vitamines B_{12} de 1000 gamma augmente généralement la tolérance du vieillard à la surcharge en glucose et diminue la glycémie deux heures après l'administration de sucre. L'effet diabétogène du prednisolone chez les personnes âgées se trouve également réduit modérément.

Il semble donc correct de considérer les vieillards comme des sujets se trouvant au stade prédiabétique: leur régime doit donc être modifié en conséquence.

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Annotation

TRANSPORT OF VITAMIN B_{12} IN MAN

The plasma transport of vitamin B_{12} is an important phase of the metabolism of this vital substance. Much has yet to be learnt, but enough is known to permit an outline of current concepts of the transport system. Some of the characteristics of the two participating transport proteins, transcobalamin I and II (TC I and II) are presented in Table I. Two other vitamin-B_{12} binders (Table I) exist and all four are glycoproteins. A further detailed account of these proteins will be published elsewhere (Hall and Finkler, 1969a).

The First Stage of Plasma Transport of Vitamin B_{12}

Intrinsic factor (IF), the transport glycoprotein of the intestine, does not pass through into the portal blood but gives up its vitamin B_{12} to the plasma transport system (Cooper and White, 1968). The absorbed vitamin B_{12} is taken up by TC II, which is the main binder of vitamin B_{12} added to or injected into plasma. This function of TC II has been studied by isolating this protein during the absorption of physiological amounts of radioactive vitamin B_{12}. TC II also takes up vitamin B_{12} when it is either mixed with or incorporated into food (Hall and Finkler, 1965, 1966a).

Absorbed vitamin B_{12} enters the blood at a slow rate: it first appears in blood 3–4 hours after a meal and reaches a peak level at 8–12 hours. Uptake of vitamin B_{12} by TC II is probably still going on at 24 hours (Hall and Finkler, 1965). If a person ingests vitamin B_{12} in food several times a day, this slow entry into the blood creates an almost constant supply of circulating TC-II–vitamin-B_{12} complex. TC-II–vitamin-B_{12} is cleared rapidly from plasma (Fig. 1) and the levels remain low, the vitamin B_{12} adsorbed to TC II probably never exceeding 20 pg. per ml. of plasma.

A good transport vehicle delivers its load with dispatch and TC II does this exceedingly well (Fig. 1): half of an injected dose of TC-II–^{57}Co-vitamin-B_{12} leaves the plasma in well under 5 minutes. Simultaneously the vitamin B_{12} accumulates rapidly in the liver and presumably the uptake is as rapid, if not as great, by other tissues. During the early stages of uptake the tissue vitamin B_{12} is probably loosely held. The rate of removal of injected TC-II–vitamin-B_{12} reported by Hom (1967) was slower than that shown in Fig. 1 because his sampling differed and he analysed a different portion of the complex disappearance curve. Hom also postulated a rapid and widespread extracellular distribution of TC-II–vitamin-B_{12}, but we could not demonstrate injected vitamin B_{12} in extracellular fluid other than plasma (Hall, 1960).

The concept of TC II as a carrier of vitamin B_{12} to cells is supported by *in vitro* studies with HeLa cells (Finkler and Hall, 1967) or with reticulocyte-rich preparations of erythrocytes (Retief, Gottlieb and Herbert, 1967). TC-II–vitamin-B_{12} is taken up much better than is vitamin B_{12} in saline. These *in vitro* studies of TC-II–vitamin-B_{12} have not been continued beyond the first few hours after uptake. Shortly after its TC-II-mediated uptake by erythrocytes, vitamin B_{12} was located mainly in the stroma and 70 per cent of that taken up by HeLa cells was in the cell membrane.

The distribution of vitamin B_{12} among tissues is uneven, possibly because cells differ in their avidity for TC-II-bound vitamin B_{12}. Such factors as blood flow through an organ,

TABLE I

PROPERTIES OF B_{12} BINDING PROTEINS IN PLASMA

	TC I	*TC II*	*PV binder*	*Foetal binder*
DEAE-cellulose, elution starts	0.12 M pH 6.0	0.035 M pH 7.0	0.04 M pH 6.5	Probably same as PV binder
CM-cellulose, elution starts	0.02 M pH 5.4	0.2 M pH 7.8	0.02 M pH 5.4	0.02 M pH 5.4
Mobility:				
pH 8.6	α_1	Fast β	α_2	α_2
pH 4.5	Anodal	Cathodal	Between TC I and TC II	Between TC I and TC II
Molecular size*	121,000	38,000	121,000	121,000
HeLa cell uptake	No	Yes	No	No
Carries endogenous B_{12}	Yes	Undetermined	No	No
Reacts with:				
Anti-TC II	No	Yes	No	No
Anti-TC I	Yes	No	Yes	Yes
Anti-saliva R	Yes	No	Yes	Yes

* Figures for molecular size are from HOM, B. and OLESEN, H. (1967). *Scand. J. clin. Lab. Invest.*, **19**, 269. All other data are from our own laboratory. The term 'PV binder' refers to the binder found in polycythaemia vera.

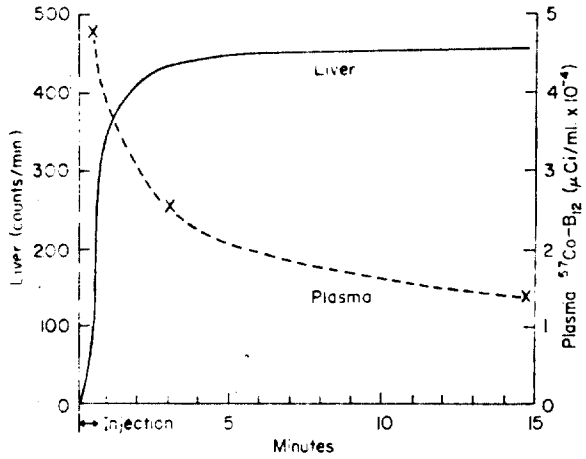

FIG. 1. Simultaneous plasma clearance and liver uptake (external counting) of TC-II-vitamin-B_{12}. The TC-II-^{57}Co-vitamin-B_{12} carried 8×10^{-3} μg. of ^{57}Co-B_{12} bound to the TC II recovered from 200 ml. of plasma and was injected intravenously into a control subject (Finkler and Hall, 1967).

area of cell surface exposed to the plasma and total number of cells or receptor sites may be equally important in determining the mode of distribution. Vitamin B_{12} entering the body bound to the TC II in portal blood first reaches the large and vascular liver, perhaps explaining the large initial hepatic uptake. Evidence from several sources, however, shows that the liver

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ultimately takes up the largest share of vitamin B_{12} no matter how it enters the body. The liver cell may, then, have a greater affinity for TC-II-bound vitamin B_{12} than other cells. It is not known why cells take up TC-II-bound vitamin B_{12} better than the free vitamin. Because TC-II–vitamin-B_{12} is retained on carboxymethylcellulose we assume that it has a net positive charge and is attracted initially by the negative charge of the HeLa cells of our *in-vitro* system.

Mouse TC II is produced in the liver (Tan and Hansen, 1968) but the source of human TC II is not known. We have found TC II in the plasma of a man who lost all of his ileum and most of his jejunum 20 years previously: TC II is not, therefore, made in the ileum, and conversion of intrinsic factor to TC II by the ileum is not the source of TC II. The plasma content of TC II is estimated to be 25 μg./l.; the TC II in normal plasma can carry an average of 986 pg. of vitamin B_{12} per ml. (Hom and Ahluwalia, 1968). Calculations based on the plasma ^{57}Co–vitamin-B_{12} after oral administration show that less than 2 per cent of the available TC II is used at any one time during absorption. According to concepts of Tan and Hansen (1968), TC II is not re-used once vitamin B_{12} has been attached to it.

Transport of Vitamin B_{12} out of Cells

TC I can carry vitamin B_{12} out of cells and probably does so. There are about 60 μg. of TC I per litre of normal plasma and this can carry 700–800 pg. of vitamin B_{12} per ml. of plasma. About half of the binding capacity of TC I is normally saturated with endogenous vitamin B_{12}, leaving half to take up added vitamin B_{12}. The terms 'endogenous' and 'native' are used to refer to vitamin B_{12} that has entered the body through natural means, and is measured in the microbiological assay for vitamin B_{12}. Vitamin B_{12} given for experimental purposes is referred to as 'added' or, when appropriate, 'radioactive'.

Vitamin B_{12} bound to TC I is removed slowly by tissues *in vivo* (Hall and Finkler, 1965; Hom, 1967), and uptake is equally poor by cells *in vitro* (Finkler and Hall, 1967; Retief *et al.*, 1967). There must, however, be some way for the body to use TC-I-bound vitamin B_{12}, since it recycles within the plasma for months (Hall, 1964). Possibly TC-I–vitamin-B_{12} is taken up, although slowly, by some tissues other than those studied. There may be a release of vitamin B_{12} initially bound to TC I, a rebinding to TC II, and then uptake by the cells.

Direct evidence suggesting that TC I carries vitamin B_{12} out of cells was reported in a single study that has since been repeated. ^{57}Co-vitamin-B_{12} bound to TC II was incorporated into HeLa cells, the culture medium containing vitamin B_{12} removed and the cells washed. They were then cultured for 48 hours in vitamin-B_{12}-free media, after which the ^{57}Co-vitamin-B_{12} that had come out of the cells was shown to be bound to TC I (Finkler and Hall, 1967). The cells may have converted TC II to TC I or may have synthesized TC I *de novo*.

There is also indirect evidence of an 'exit' function for TC I. After injection of TC-II-^{57}Co-vitamin-B_{12}, Hom (1967) found no TC-I-^{57}Co-vitamin-B_{12} in the plasma at 10 minutes, though it appeared in the 4-hour sample of plasma. The time required for the appearance of this complex is compatible with the concept of intracellular synthesis of TC I. Slow transfer of vitamin B_{12} from TC II to TC I within the plasma is an alternative, although unsupported, explanation. Stahlberg (1964) has shown that most of the endogenous vitamin B_{12} in plasma is methylcobalamin. Since most of the endogenous vitamin B_{12} in the plasma

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is bound to TC I, TC I must carry methylcobalamin in plasma. Putting together the observations one is left with the concept that TC I carries methylcobalamin out of cells.

Leucocytes are often mentioned as a source of TC I. Gräsbeck postulates that the vitamin B_{12}-binding protein in leucocytes is converted to TC I by the addition of sialic acid molecul to the protein (Stenman, Simons and Gräsbeck, 1968); this is an attractive hypothesis: sever vitamin-B_{12} binders in the body closely resemble TC I and such a conversion might occ in more than one type of tissue.

Intermediate Stages of Transport of Vitamin B_{12}

We do not know what happens to vitamin B_{12} within the cell or where it is convert into its functioning forms. Intermediate plasma transport seems to occur as methylcobalam via TC I but where from, where to, and for what purpose remain uncertain. TC II ma participate in intermediate transport and this may remain undetected because of the rapi clearance of TC-II–vitamin-B_{12}. If vitamin B_{12} ever comes out of cells in free form, it woul bind to TC II. Whatever the mechanism, body vitamin B_{12} is certainly re-used.

Plasma Transport of Vitamin B_{12} in the Foetus

Kumento, Lopez, Luhby and Hall (1967) have found a new binder in all foetal bloo examined and in small amounts in some maternal blood: it disappears from foetal bloo within a few weeks, it does not carry endogenous vitamin B_{12} and it does not promot vitamin-B_{12} uptake by HeLa cells. Other properties are given in Table I. Foetal bloo contains TC I and II but the latter appears to carry more endogenous vitamin B_{12} tha adult TC II.

Abnormal Plasma Transport of Vitamin B_{12}

Vitamin-B_{12} transport is disturbed in some diseases, but so far no harmful effects can b attributed to these abnormalities. They include: (1) an increased TC I in all of the chroni myeloproliferative states, most striking in chronic granulocytic leukaemia; (2) a vitamin-B_{1} binder in the blood of patients with polycythaemia vera (PV) (Table I) which differs fron TC I and II but possibly not from the foetal binder (Hall and Finkler, 1969b); (3) a decrease TC II in chronic myeloproliferative states and in some patients with pernicious anaemia (Hal and Finkler, 1966b), and (4) a normal amount of TC I which is carrying subnormal amount of vitamin B_{12} in pernicious anaemia.

The PV binder and the increased TC I of chronic granulocytic leukaemia probabl originate from the same, as yet unknown, type of abnormality. The differences betwee these two binders, which are probably minor, may be due to differences in the cell of origin the PV binder might be the distorted product of a disturbed cell, which could explain it failure to carry endogenous vitamin B_{12}. Alternatively, perhaps it is produced by a cel which is itself deficient in vitamin B_{12}.

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Measurement of the Amounts of the Individual Vitamin B_{12} Binding Proteins in Plasma

II. Abnormalities in Leukemia and Pernicious Anemia

By CHARLES A. HALL AND ALEXANDER E. FINKLER

THE HIGH CAPACITY of serum from patients with chronic myelogenous leukemia (CML) to bind vitamin B_{12} has interested many investigators.[1-4] The most likely cause of this increased binding capacity has been considered to be an increase in the protein that transports the endogenous B_{12}, transcobalamin I (TC I) of our terminology.[5,6] Qualitative changes which could increase its capacity have also been considered.[1] In vivo work in our laboratory showed that there may also be an abnormality of transcobalamin II (TC II) in CML.[7] Therefore, we devised the technic for measuring TC I and TC II described in Part I of this presentation and applied them to the problem of the binding pattern in disease. This work has been presented in preliminary form.[8]

RESULTS

The binding patterns after the addition of 300 pg. of $Co^{57}B_{12}$ per ml. of plasma are given in Table 1. There was no binding to TC II in any of the plasma from the 3 patients with CML, but more than the usual amount was bound to TC I. In the one patient tested before and after successful treatment with busulfan, there was a shift toward a more normal pattern. Patients in partial or complete remission and those with acute myelogenous leukemia (AML) showed abnormally large amounts bound to TC I and less than normal binding to TC II. An intermediate pattern was also seen in pernicious anemia (PA) in relapse.

With the suggestion that the amount of TC I might be increased in these forms of leukemia, the binding capacity was tested by adding "above capacity" amounts of B_{12}. Figure 1 shows the pattern at 3 levels of B_{12} in subject G. W. with severe CML. There was no TC II binding. At 10 ng./ml. a large amount was bound to TC I. At 20 ng./ml. no more was bound to TC I but some appeared in the main protein region of the chromatogram. This pattern was compared with that of subject N. W. with PA (Fig. 2). In this case there was also no significant binding to TC II, but there was a sharp contrast in respect to TC I.

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Table 1.—*Co^{57} Cyanocobalamin Bound to Plasma Fractions after 300 pg. ml.*

Type of Subject	Per Cent Bound	pg. ml. Plasma TC I	Main Protein Peak	TC II
Mean of 4 controls	90	26	12	120
CML relapse—A.M.	79	170	0	0
A.S.	80	200	0	0
C.W.	99	210	0	0
CML relapse—A.M.	79	170	0	0
Remission—A.M.	82	95	0	54
CML—complete or partial remission mean of 4 patients	88	93	0	52
AML relapse mean of 3 patients	96	120	0	55
PA relapse mean of 4 patients	90	58	0	99

At the 10 ng./ml. level relatively little could be added to TC I, while large amounts were bound in the main protein region. It appeared then that, as presented in Part I, the technic used gave a rough measurement of the binding capacities of TC I and TC II and perhaps the amount of each substance. This approach was incorporated in the studies of 5 subjects given in Table 2. We interpreted the results as follows: In CML, TC II was absent, diminished or nonfunctioning. TC I was either much increased or the binding capacity per molecule was increased. TC I or its capacity was increased to a lesser degree in AML. In the patient with PA, TC II or its function was diminished but TC I had a normal capacity.

Discussion

The demonstration of the increased capacity of TC I to bind added vitamin B_{12} in CML is a direct confirmation of the earlier conclusions of Miller and Sullivan[5] and Mendelsohn, Watkin, Horbett and Fahey.[6] There may be an absolute increase in the amount of this substance or there may be a qualitative abnormality of TC I, as suggested by Beard, Pitney and Sanneman,[1] which increases its binding capacity. The diminished, absent, nonfunctioning or inhibited TC II has not been reported previously. The intermediate type of abnormal binding pattern described here in AML was to be expected since some abnormality of binding has been described in several of the myeloproliferative syndromes.[1-4,9-10] The inability of TC II to take up normal amounts of B_{12} in patients with PA requires further study.

TC II has a function in the normal human B_{12} metabolism.[11] TC II bound B_{12} was taken up by intact tissues more readily than TC I bound B_{12}.[11] We also showed that TC II bound B_{12} was taken into HeLa cells in tissue culture but free B_{12} or TC I bound B_{12} was not.[12] Therefore, the loss of function of TC II in CML may have important consequences.

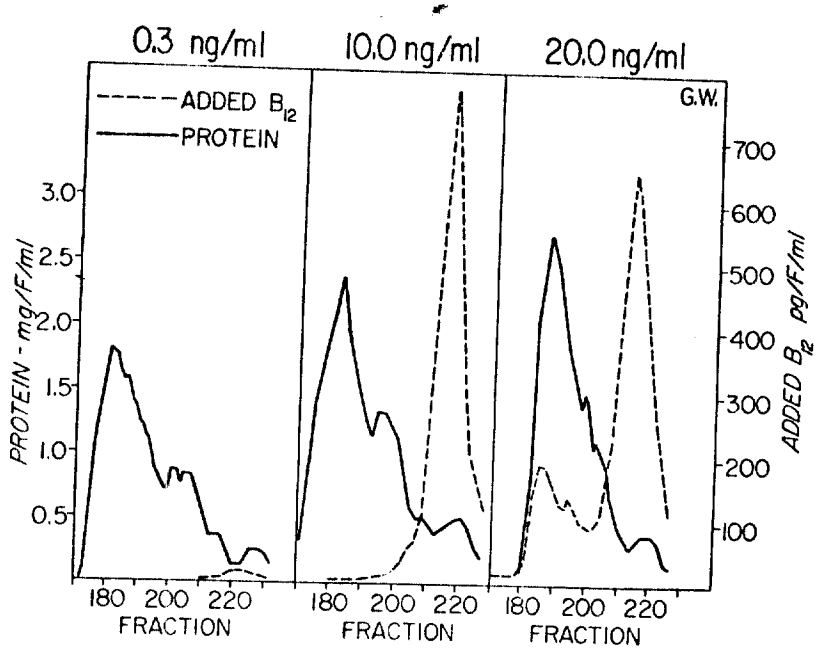

Fig. 1.—Comparison of three levels of cyanocobalamin added to samples of plasma from subject G. W. with CML. There was no binding in fractions below #170.

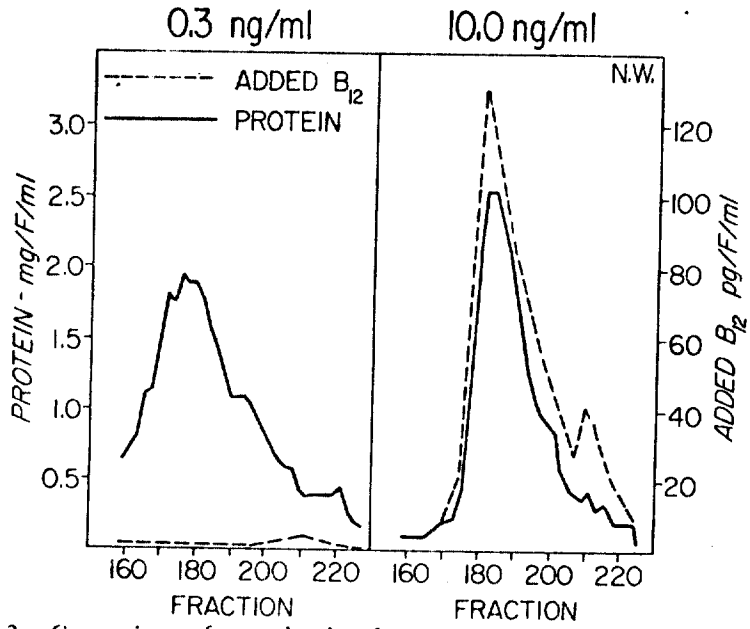

Fig. 2.—Comparison of two levels of cyanocobalamin added to samples of plasma from subject N. W. with pernicious anemia. Binding in the fractions below #160 was too slight to be shown by the scale used.

Table 2.—*Co^{57} Cyanocobalamin Bound to Plasma Fractions with Increasing Amounts Added*

Type of Subject and Amount Added in ng./ml. Plasma		Per Cent Bound	pg./ml. Plasma TC I	Main Protein Peak	TC II
Control	0.3	95	28	0	140
A.H.	5.0	55	350	1,000	320
	10.0	52	220	2,700	560
CML	0.3	99	210	0	0
G.W.	10.0	87	8,000	0	140
	20.0	72	9,200	2,000	150
CML	0.3	79	170	0	0
A.M.	10.0	87	7,500	0	450
AML	0.3	90	140	0	28
C.H.	10.0	56	1,800	1,100	580
PA	0.3	91	43	0	24
N.W.	10.0	64	380	2,300	0

Any discussion of the causes of the abnormalities of binding observed in chronic and acute myelogenous leukemia is pure speculation. They may be closely related to the cause of the disease or may be secondary or even compensatory in nature. The increase in TC I could be the result of the production of an abnormal protein as in multiple myeloma. In leukemia the evidence permits postulation of a reciprocal relationship between TC I and II; that is, when one rises the other falls. This does not seem to be the case in PA where apparently TC II can be diminished or nonfunctioning without any change in TC I.

There are some important points to be considered when the findings of the present study are used to interpret the dialysis technic of measurement of total B_{12} binding by serum or plasma. As discussed in Part I, TC II was the principal or primary binder in normal plasma when small amounts of B_{12} were added. Some other protein bound any additional B_{12} at about the 1.0 ng./ml. level and above. The primary binder was TC I in the plasma of CML at low levels of B_{12}, and it bound B_{12} as avidly as the TC II of normal plasma. The secondary binders accompanying the bulk of the plasma proteins did not become important until above the 10.0 ng./ml. level in CML plasma. In AML and in CML in remission, both TC I and TC II functioned as primary binders, and the level of B_{12} at which proteins of the main protein peak became important as secondary binders was greater than that of normal plasma. It is clear that plasma binding of B_{12} is not a single process by a single substance but is the balance of binding by several substances and that the balance is a function of both the amount of B_{12} added and the source of the plasma.

Summary

The vitamin B_{12} binding capacities of transcobalamin I and II were evaluated in plasma from patients with diseases known to affect the plasma binding.

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In CML in relapse, the capacity of TC II was very much diminished or absent. The capacity of TC I was very much increased. There was a lesser degree of both abnormalities during remission and in AML. There was either diminished or absent binding to TC II in PA but the capacity of TC I was not altered.

SUMMARIO IN INTERLINGUA

Le capacitates de ligar vitamina B_{12} de transcobalamina I e transcobalamina II esseva evalutate in le plasma de patientes con morbos cognoscitemente afficiente le ligation intraplasmatic. In chronic leucemia myelogene in recidiva, le capacitate de transcobalamina II esseva grandemente diminuite o absente. Le capacitate de transcobalamina I esseva grandemente augmentate. Ambe le mentionate anormalitates esseva minus pronunciate in remission e in acute leucemia myelogene. In anemia perniciose, le capacitate ligatori de transcobalamina II esseva reducite o absente, durante que le capacitate de transcobalamina I non esseva alterate.

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Vitamin B_{12} levels in erythrocytes in normal subjects and in pernicious anaemia

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SYNOPSIS A reliable method is described for the assay of vitamin B_{12} activity in erythrocytes using *Lactobacillus leichmannii*. The results are shown for age, sex, and blood group in normal subjects, and before, during, and after therapy in vitamin B_{12} deficiency anaemia.

The normal range is 85-225 pg/ml (mean = 155 ± 35 pg/ml). Raised levels were found in the newborn, and subnormal levels in subjects aged 70 years and over and in males aged 10 to 19 years. There were no differences in erythrocyte vitamin B_{12} levels between the ABO or Rhesus blood groups.

In pernicious anaemia low levels are found before treatment (mean = 91 pg/ml), followed by a rapid rise during vitamin B_{12} therapy (mean peak = 482 pg/ml), and falling more slowly towards normal after the initial haematological response.

Most of the work on vitamin B_{12} levels previously reported in the literature has been performed either on serum or on plasma. Since vitamin B_{12} is essentially an intracellular substance, however, it is particularly relevant to know something of its concentration within cells as well. Erythrocytes were the obvious choice for this purpose since they are the most easily obtained of all body cells, and because it is known that various changes occur in them in pernicious anaemia (Price-Jones, 1929; Israels, 1939; Scott, 1939).

Levels in normal whole blood (Table I) have been reported by Couch, Olcese, Witten, and Colby (1950), Sobotka, Baker, and Ziffer (1960), and by Rosenthal and Brown (1954), but these are open to ambiguous interpretation. In unwashed normal erythrocytes Kelly and Herbert (1967), using a radioactive assay, obtained a range of 72 to 520 pg/ml, and Biggs, Mason, and Spray (1964), using the lactobacillus, found 110-500 pg/ml.

Biggs *et al* (1964) considered that washing the erythrocytes was unnecessary, despite the fact that one patient in their series, who was suffering from myeloid leukaemia, showed a fall in the vitamin B_{12} content of the red cells from 745 pg/ml to 440 pg/ml after two washings of the cells in saline.

In normal subjects vitamin B_{12} levels in erythrocytes are usually lower than those in plasma, as shown in Table I. Thus it is preferable to remove traces of plasma from erythrocytes before assay to ensure the absence of any error from this source. Also, blood from patients receiving vitamin B_{12} therapy was to be tested in the present survey, and experiments on blood taken within 60 hours after the injection of 1,000 μg cyanocobalamin showed (Table II) that washing the cells reduced their vitamin B_{12} level by up to 400%, depending on the plasma B_{12} levels. The reduction in normal subjects who were not receiving vitamin B_{12} was up to 40 pg/ml, ie, up to 20%. These results confirmed the need to wash the cells before assay.

It will be noticed from Table I that in only two of the reports were the erythrocytes washed before examination (Sobotka *et al*, 1960; Kato, 1958). In both of these communications only a relatively small number of subjects were examined (three and 20 respectively) and it is clearly desirable that to establish the normal range of vitamin B_{12} concentration in erythrocytes accurately a larger series of investigations is needed.

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Reference	No. of Patients	Source	Vitamin B_{12} Level (pg/ml)	
			Range	Mean
Couch *et al* (1950)	10	Whole blood	600-1,400	800
Sobotka *et al* (1960)	3	Washed RBCs	140-185	165
		Whole blood	260-425	332
		Plasma	320-620	513
Rosenthal and Brown Jr. (1954)	—	Whole blood		260
Biggs *et al* (1964)	50	Unwashed RBCs	110-500	213
		Plasma	150-1540	603
Kelly and Herbert (1967)	50	Unwashed RBCs	72-520	209
		Plasma	196-584	392
Kato (1958)	20	Washed RBCs	135-230	195
		Plasma		362
Present work	256	Washed RBCs	80-280	155
		Plasma	140-920	310

Table I *Vitamin B_{12} activity in normal subjects*

Subject	Plasma	Vitamin B_{12} Level (pg/ml)				
		Number of Erythrocyte Washes				
		Nil	1	2	3	4
Pernicious anaemia						
1	600	380	280	260	240	—
2	2,100	1,800	780	530	500	—
3	2,600	3,100	1,800	960	800	800
4	3,800	2,600	1,200	240	166	166
5	1,200	—	430	270	—	270
Normal						
1	280	220	190	184	—	
2	540	260	220	220	220	
3	760	108	112	112	108	
4	360	160	140	140	—	
5	292	160	—	144	—	
6	230	180	—	160	—	

Table II *The effect of washing the erythrocytes on vitamin B_{12} level in cases of pernicious anaemia on intramuscular Cytamen and in normal subjects*

Materials and Methods

The method used was based upon a modification by Biggs *et al* (1964) of Spray's technique (1955) for the estimation of vitamin B_{12} in serum. After the investigation of various factors involved, including the extraction of B_{12} from unhaemolysed cells and from cells haemolysed after thawing from −20°C, buffer *p*H, various concentrations of cyanide, interference by protein, and length of incubation, some alterations were made to the original method. These are included in the following section, together with the reasons for introducing the changes.

METHOD

Ten ml blood was collected, by venepuncture, into heparin. Plasma was separated by centrifuging and the red cells were washed twice in 0·9% saline and centrifuged. The buffy layer was removed with the supernatant fluid. (Three washes were required if the patient was receiving vitamin B_{12}.) The cells were then stored at −20°C until the assays were performed, usually within four weeks.

Extracts of the erythrocytes were prepared in 1 oz universal containers by adding 2 ml of washed erythrocytes to 2 ml 0·4 M-acetate buffer (*p*H 5·2), 16 ml deionized water, and 1 drop of 0·5% (w/v) sodium cyanide and mixing. The samples were then autoclaved at 110° for 30 minutes (Spray, 1955) and centrifuged. From each sample amounts of clear supernatant fluid, each of 5 ml, were placed into each of three 5 in. × $\frac{5}{8}$ in. test tubes. Standards of 0, 25, 50, 100, 200, 300, and 400 pg of cyanocobalamin were also prepared. To each was added 5 ml of double-strength assay medium (Difco) and the tubes were capped and placed in a water bath at 60°C for 30 minutes to maintain sterility. After cooling to 37°C, 1 drop of washed suspension of actively growing *Lactobacillus leichmannii* was added to each tube. The tubes were incubated at 37°C for 72 hours and finally titrated with 0·2 N sodium hydroxide using phenolphthalein as indicator.

The medians of the test titrations were read off a graph made from the standards on semi-log paper.

THE TEST ORGANISM (*LACTOBACILLUS LEICHMANNII*)

An important factor in obtaining reliable assays was found to be in the use of an actively growing lactobacillus (Spray, 1955). This was obtained by repeated subculture of the organism until a six- to eight-hour growth could be seen to be abundant. A minimum of two subcultures is usually needed to achieve this. Adequate washing of the lactobacillus is also required to remove all vitamin B_{12} present in the culture medium (Spray, 1955) and thereby improve the 'sensitivity' of the method. Thus traces of vitamin B_{12} inoculated into the assay tubes gave a higher blank reading and masked small amounts of the vitamin present in the sample being assayed. Good results, with very low blank titrations, were obtained by the 'three-tubes transfer' technique of washing. The actively growing culture is centrifuged at 3,000 rpm for five minutes, the supernatant decanted, and the tube drained by inversion on blotting paper. The organisms at the bottom of the tube were removed to a clean tube by adding 1 or 2 drops of assay media directly onto the deposit. The tube was filled with assay media, mixed, and centrifuged. Washing was then repeated twice. The washed organisms were then diluted with assay medium until the suspension matched approximately Brown's opacity tube no. 1 (400,000 organisms per ml) and were ready for inoculation.

Age, Sex, and No. of Subjects	*Erythrocyte Vitamin B_{12} Activity (pg/ml)*					
	Mean ± SD		*Range*	*Em*	*t*	*P*
At birth						
Males (3)	172	28	144-200	16	1·06	0·4
Females (6)	190	41	144-250	17	2·11	0·08
M & F (9)	184	36	144-250	12	2·3	0·05
10-19 Years						
Males (6)	109	41	80-182	16	2·7	0·05
Females (6)	162	25	132-200	8·9	0·78	0·5
M & F (12)	146	42	80-200	12	0·75	0·5
20-29 Years						
Males (8)	152	22	100-168	7·7	0·04	0·97
Females (8)	160	23	124-196	8	0·63	0·6
M & F (16)	156	22	100-196	5·5	0·18	0·9
30-39 Years						
Males (7)	141	27	111-170	10	1·4	0·3
Females (5)	156	10	142-164	4·2	0·24	0·9
M & F (12)	147	22	111-170	6·3	1·27	0·3
40-49 Years						
Males (8)	169	42	110-232	15	0·94	0·4
Females (6)	149	36	114-180	15	0·04	0·97
M & F (14)	160	36	110-232	9·5	0·5	0·7
50-59 Years						
Males (5)	163	27	138-200	12	0·7	0·6
Females (5)	158	21	124-178	9·2	0·3	0·8
M & F (10)	160	23	124-200	7·1	0·7	0·5
60-69 Years						
Males (2)	134	42	104-164	30	0·7	0·6
Females (9)	159	20	120-196	6·7	0·6	0·6
M & F (11)	154	24	104-196	7·3	0·01	0·99
70 & Over						
Males (1)	128	—	—	—	—	—
Females (8)	138	22	112-180	7·8	2·2	0·06
M & F (9)	137	21	112-180	7	2·57	0·05
All Ages						
Males (38)	151	35	80-232	5·7	0·7	0·5
Females (55)	158	27	112-250	3·6	0·83	0·5
M & F (93)	155	31	80-250	3·2	—	—

Table III *Vitamin B_{12} activity related to age and sex*

RECOVERY OF ADDED COBALAMIN FROM ERYTHROCYTES

Assays were made on washed erythrocytes to which vitamin B_{12} had been added in various concentrations. Recoveries obtained were 100% ± 6%.

ACCURACY OF THE METHOD

Assays were made on 50 samples of human erythrocytes. Fifty-four per cent of the results agreed within ± 5% of the initial levels and 70% were within ± 10%. The standard deviation was ± 10%.

Results in Normal Subjects

The mean and standard deviation of vitamin B_{12} activity in erythrocytes in 256 apparently normal subjects was 155 ± 35 pg/ml. The complete range was 80 to 280 pg/ml but 95·5% of readings obtained from the normal population had levels between 85 and 225 pg/ml.

The results for age and sex in 93 subjects are shown in Table III and the results relating to blood group in Table IV. The age group 0 to 9 years was not examined, apart from nine newborn infants. There was a slightly significant rise above the mean ($P = 0·05$) at birth and slight falls below the mean age 10-19 years in males ($P = 0·05$) and also at age 70 years and over ($P = 0·05$). There were no statistically significant differences between the remaining age groups of 10 to 69 years. There were also no significant differences between the various ABO or Rhesus blood groups.

Furthermore there was no apparent relationship between plasma and erythrocyte vitamin B_{12} activity in normal subjects (Fig. 1); thus plasma levels could be high with low normal erythrocyte levels. However, greater variability was observed in the plasma levels, ie, 140 to 920 pg/ml, than in the erythrocyte levels, 80 to 280 pg/ml, which suggests that the erythrocyte level is a more critical indication of the cell content of vitamin B_{12} than is the plasma level; the latter level probably reflects the nature of the subject's diet.

Blood Group	Rh	No. of Subjects	Mean	SD	Range	Em	t	P
O	Positive	41	161	36	96-250	5·6	1·08	0·3
O	Negative	26	159	43	82-260	8·5	0·5	0·7
A	Positive	46	155	36	88-220	5·2	0	1·0
A	Negative	25	166	41	112-280	8·2	1·3	0·3
B	Positive	18	145	35	86-216	8·3	1·2	0·3
AB	Positive	7	151	14	126-160	5·3	0·76	0·95
All ABO groups								
Rh positive		112	150	36	88-250	3·4	1·47	0·2
Rh negative		51	162	36	82-280	5·0	1·4	0·2
O positive and negative		67	160	39	82-260	4·7	1·06	0·3
A positive and negative		71	159	38	88-280	4·5	0·9	0·4
All groups		163	155	37	82-280	2·9	—	—
Normal controls plus blood donors		256	155	35	80-280	2·2	—	—

Table IV *Vitamin B_{12} activity in erythrocytes from blood donors*

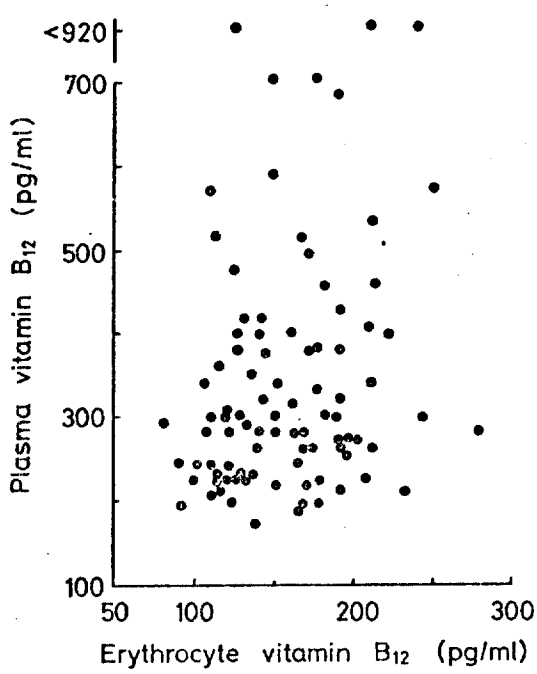

Fig. 1 *The relation of the plasma and erythrocyte vitamin B_{12} levels in normal subjects.*

Erythrocyte Vitamin B_{12} Activity in Pernicious Anaemia

The diagnosis of pernicious anaemia was confirmed in each case by the peripheral blood picture, sternal marrow examination, histamine-fast achlorhydria, and plasma vitamin B_{12} level of 102 pg/ml or less. None had clinical evidence of steatorrhoea and no patients with postgastrectomy vitamin B_{12}-deficiency anaemia were included in this series.

The relatively high initial plasma B_{12} level in the patients with pernicious anaemia is partly due to low blank titrations causing an increased sensitivity of the method for vitamin B_{12}. (High blank titrations may mask small quantities of B_{12}.) It is also partly due to selection of patients with pure B_{12} deficiency and the exclusion of those with combined folate or iron deficiency. The range of plasma B_{12} values for the whole series was 20-102 pg/ml (mean = 50 pg/ml).

Progress was assessed by the packed cell volume (PCV) readings performed on the Hawksley microcentrifuge and by haemoglobin estimation (100% = 14·8 g%). All responded typically to vitamin B_{12} therapy.

The behaviour of the erythrocyte B_{12} levels is shown in Fig. 2 and Table V, and the main haematological details are presented in Table VI. Patients 1, 2, and 3 received Cytamen, 1,000 μg on alternate days for one week, then 1,000 μg every month. Patients 4 and 5 received 100 μg twice weekly during the period of surveillance.

The vitamin B_{12} activity in the erythrocytes was low (mean = 87 pg/ml) before therapy. After intramuscular vitamin B_{12} therapy there was a rapid rise which coincided approximately with the reticulocyte response. The peak level observed (mean = 427 pg/ml) during the period of surveillance was approximately five times the initial level. This is a highly significant rise statistically ($P = <0{\cdot}001$) both in relation to the level before treatment and to the normal mean level of 155 pg/ml.

The vitamin B_{12} concentration then returned gradually after this initial peak towards normal limits over a period of one to two months. At the end of the haematological and B_{12} response (approximately 70 days) the level was 188 pg/ml, which, although above normal, was not significantly so ($P = 0{\cdot}06$).

The patterns of change were similar in all the patients, and those observed in case 1 are described in detail as an example. The initial Hb was 4 g%, reticulocyte count 2·2%, and vitamin B_{12} activity in plasma was 98 pg/ml and in erythrocytes 104 pg/ml. In response to a dosage of cyancobalamin intramuscularly of 1,000 μg on alternate days, the reticulocyte count rose to a peak of 26·4% on the sixth day. On this same day a maximum level of 520 pg/ml of B_{12} was observed in the erythrocytes. The reticulocyte count then declined to 10·2% on the 12th day but at this stage the erythrocyte B_{12} level still remained high (450 pg/ml). The reticulocyte count continued to fall and reached 0·4% by the 29th day, but the erythrocyte B_{12} level fell more slowly and was still 270 pg/ml on this day, and

the erythrocyte B_{12} level did not, in fact, return to normal (190 pg/ml) until the 91st day. The haematological response was good and the Hb rose steadily throughout to reach 9·6 g% on the 29th day and 13·3 g% on the 91st day.

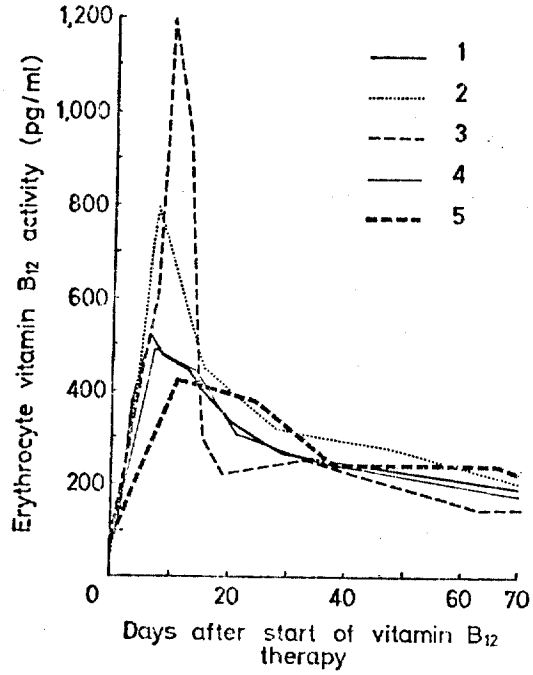

Fig. 2 *Erythrocyte vitamin B_{12} levels in pernicious anaemia during therapy.*

Case No.	Erythrocyte B_{12} Levels (pg/ml)				
	Initial	Maximum Observed	Day	Final	Day
1	104	520	6	190	70
2	54	800	7	206	69
3	92	1,200	9	156	70
4	76	490	7	170	75
5	98	420	11	204	70
Mean	85	686	8	185	71
P	<0·001	<0·001		0·06	

Table V *Erythrocyte vitamin B_{12} levels in pernicious anaemia before, during, and after therapy with B_{12}*

Discussion

In obtaining reliable results in the microbiological assay of vitamin B_{12} by the use of *Lactobacillus leichmannii* the two most important factors were found to be the use of an actively growing organism and the thorough washing to remove traces of exogenous vitamin B_{12} from the organism before inoculation. Neglect of these two steps could cause irregularities in the results. Thus, Biggs *et al* (1964) and Kelly and Herbert (1967) have reported vitamin B_{12} activities in unwashed erythrocytes in which the upper levels in normal subjects were found to be at least twice as high as the upper limits reported in washed red cells by Sobotka *et al* (1950) and Kato (1958) and in those reported in this communication (Table I). The difference is most probably due either to vitamin B_{12} in the film of plasma of unwashed red cells, which is loosely attached to the red cell membrane, or, possibly, that dialysable vitamin B_{12} may be present in the cell or its membrane. The increased levels of vitamin B_{12} activity found in the newborn may be due to the large numbers of immature cells present. The significantly subnormal levels in subjects over the age of 70 years may be related to the fall in plasma levels of the vitamin which is known to occur in the elderly (Chow, Wood, Horonick, and Okuda, 1956; Boger, Wright, Strickland, Gylfe, and Ciminera, 1955).

The subnormal levels in males aged 10 to 19 years may be due to the rapid rate of growth at this age, when the haemopoietic tissues compete with other tissues for vitamin B_{12}, folic acid, and iron. Other factors must be involved, however, since the levels in females of this age were normal.

In pernicious anaemia the concurrence of a rise in vitamin B_{12} activity in the erythrocytes with the reticulocyte response suggests that a factor may be common to both, ie, the presence of increased quantities of assayable vitamin in immature or developing cells. In this connexion it should be recalled that Schilling (1964) noted

Case No.	Age (yr)	Sex	Days Observed	Dose of CN-B_{12} In First Week (µg)	Haemoglobin (g%)		Reticulocyte Response		Plasma B_{12} Level (pg/ml)	
					Initial	Final	Maximum (%)	Day No.	Initial	Final
1	72	F	91	3,000	3·9	13·3	26·4	6	98	5,000
2	63	F	69	3,000	5·8	14·8	24·0	6	66	400
3	64	F	96	2,000	5·2	14·0	16·0	9	70	440
4	61	F	114	2,000	7·7	14·0	16·0	7	100	280
5	76	F	94	100	8·9	13·4	—	—	76	376

Table VI *Details of responses of five cases of pernicious anaemia during initial two to three months on vitamin B_{12} therapy*

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that the uptake of radioactive B_{12} by immature erythrocytes coincided with the reticulocyte count. It is also of interest, however, that the increased non-radioactive vitamin B_{12} activity still persists from some time after the reticulocyte count has returned to normal. This suggests that some cells which show no visible sign of immaturity on routine screening (eg, the presence of reticulin, polychromasia, or stippling) may, nevertheless, still show microbiological signs of incomplete maturity, as demonstrated by persisting raised vitamin B_{12} activity. It is also pertinent to speculate whether this means that the fully mature cell loses some vitamin B_{12} during maturation, or whether the vitamin is gradually incorporated into the protein structure of the erythrocyte in such a way as to make it no longer releasable by simple autoclaving in the presence of cyanide. In this connexion it has been shown by Mooney and Heathcote (1967) that only 3% of the total vitamin B_{12} present in casein is releasable by autoclaving at 10 lb pressure for 30 minutes, and it is reasonable to infer that similar binding may occur during the complete maturation of other proteins, including erythrocytes, as well. This point requires further study.

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ERYTHROCYTE VITAMIN B_{12} LEVELS IN PREGNANCY

BY

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Summary

In normal pregnant women vitamin B_{12} levels in erythrocytes tend to be subnormal despite a normal serum vitamin B_{12} and the absence of anaemia.

IN pregnancy overt megaloblastic anaemia due to folate deficiency occurs in approximately 3 per 100 women, and serum folate levels tend to fall during the last trimester (Britton, 1963; Willoughby and Jewell, 1966). Vitamin B_{12} deficiency anaemia is very rarely found at this time (Mollin and Ross, 1954) and serum vitamin B_{12} levels are usually normal, even when a megaloblastic anaemia is present (Girdwood, 1954; Metz *et al.*, 1960). Little is known, however, about the behaviour of the erythrocyte levels of vitamin B_{12} during pregnancy. A study was therefore undertaken and is the subject of this report.

METHOD

Vitamin B_{12} levels were assayed in plasma according to the method of Spray (1955) and in erythrocytes by the method reported by Harrison (1970). In each case cyanide extraction was performed and the *Lactobacillus leichmannii* was used as the assay organism. Serum folate activity was measured by using *Lactobacillus casei* (Waters *et al.*, 1961).

RESULTS

The erythrocyte vitamin B_{12} levels in 13 normal pregnant subjects ranged from 104 to 180 pg. per ml., with a mean of 133±S.D. 20 pg. per ml. (Table 1). This level was highly significantly low ($P = 0{\cdot}005$) compared with the normal mean found in non-pregnant women of 155±35 pg. per ml. (range = 100–220 pg. per ml.).

In one normal pregnant woman who was receiving prophylactic folic acid—and for this reason is not included in the above series—the erythrocyte vitamin B_{12} level was 190 pg. per ml. In two patients with untreated anaemia due to folate deficiency the mean erythrocyte B_{12} level in 4 samples was 88±8·5 pg. per ml., which was also significantly below normal ($P = <0{\cdot}01$). These levels were similar to those found in non-pregnant subjects with folate deficiency anaemia (Harrison, 1971).

The serum vitamin B_{12} levels in all 13 subjects were normal and ranged from 184 to 376 pg. per ml. (normal level, above 140 pg. per ml.). The serum folate levels were also normal and ranged from 8·3 to 15·5 ng. per ml. (normal, 7·5 to 25 ng. per ml.). Haemoglobin levels were normal for pregnancy, ranging from 12·2 to 14 g. per 100 ml.

DISCUSSION

The tendency of vitamin B_{12} levels in the erythrocytes of normal pregnant women to be subnormal might suggest a subclinical deficiency of this vitamin. However, this is highly unlikely because plasma B_{12} levels are normal and liver stores of this vitamin (4 mg. on average) are more than enough to supply both maternal and fetal requirements during pregnancy. On the other hand the body stores of folate are only sufficient for about four months, and low serum folate levels are found after only three weeks on a folic acid-free diet (Herbert, 1962). With the additional burden of providing folate for the developing fetus, folate stores may be depleted easily. In normal women as pregnancy progresses the folate level falls in the serum (Willoughby

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TABLE I

Plasma and erythrocyte vitamin B_{12} and serum folate levels in normal pregnant women

Case no.	Age (years)	Parity	Duration of gestation (weeks)	Vitamin B_{12} (pg./ml.)		Serum folate (ng./ml.)	Haemoglobin (g./100 ml.)
				Plasma	Erythrocyte		
1	19	0	26	370	180	12·6	13·8
2	22	1	30	260	124	9·6	13·6
3	20	0	27	242	140	13·5	13·8
4	31	4	32	196	104	8·5	13·2
5	19	1	34	376	120	9·1	12·6
6	17	0	30	244	148	12·3	13·5
7	23	0	28	320	150	15·5	13·1
8	21	2	28	200	132	8·3	14·0
9	24	1	24	300	132	10·6	13·5
10	25	0	28	440	140	8·3	13·1
11	22	0	32	184	120	12·7	12·2
12	20	0	30	280	110	13·2	13·6
13	20	1	32	250	132	10·6	13·9

and Jewell, 1966) and in the erythrocytes (Willoughby and Jewell, 1968). Megaloblastic changes in the marrow may occur in the absence of anaemia during pregnancy (Drury and Coyle, 1960), suggesting a subclinical deficiency of folate.

In patients with anaemia due to folate deficiency, who have a low erythrocyte B_{12} and a low serum folate concentration, both these levels and the anaemia may be corrected by folic acid alone (Harrison, 1971).

The subnormal levels of vitamin B_{12} in erythrocytes observed during apparently normal pregnancy are probably secondary to a subclinical deficiency of folate, or to a metabolic change which interferes with the uptake of haemopoietic factors by erythrocyte precursors. A maternal folate deficiency, however, is not necessarily reflected in the fetus, for although 21 per cent of apparently normal and well-nourished pregnant women had low serum folate levels, their fetuses had high plasma and erythrocyte folate levels (Avery and Ledger, 1970). Baker *et al.* (1958) also noted that serum folate levels are higher in infants than in their mothers. Thus the fetal needs for folate appear to take precedence over the maternal ones. This also appears to occur with vitamin B_{12} since the serum levels of this vitamin are higher in infants than those found in their mothers (Baker *et al.*, 1958).

These findings support the possibility of intrinsic metabolic factors causing fetal needs for folate to take preference over maternal ones. In many cases, when folate reserves are low this causes obvious clinical deficiency, but subclinical deficiency of folate may be commoner than previously suspected in pregnancy and this appears to be reflected in subnormal levels of vitamin B_{12} in erythrocytes.

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1196. *The Chemistry of Vitamin* B_{12}. *Part IV.*[1] *The Thermodynamic* trans-*Effect*

By G. C. Hayward, H. A. O. Hill, J. M. Pratt, N. J. Vanston, and R. J. P. Williams

Equilibrium constants are reported for several ligand replacement reactions of cobalamins and cobinamides (see Figure) and are interpreted in terms of the thermodynamic *trans*-effect of the ligand which is not replaced. It is shown that as this ligand is changed in the order H_2O, NC^-, $HC{\equiv}C^-$, $H_2C{=}CH^-$, CH_3^-, so the *trans* cobalt–cyanide bond becomes less stable relative to the cobalt–benziminazole bond which in turn becomes less stable relative to the cobalt–water bond. The work involved the identification of certain cobalamins and cobinamides.

This Paper presents a study of equilibria involving the binding of axial ligands to the cobalt of vitamin B_{12} (see Figure). The results are discussed in terms of the thermodynamic *trans*-effect of one ligand X on the equilibria for the replacement of other possible axial ligands Y by Z. The corrinoids are particularly well suited for the study of this effect in general for the following reasons.

(1) Octahedral co-ordination eliminates the uncertainty concerning the binding of further ligands which is inherent in studying square-coplanar complexes.

(2) The equatorial ring prevents *cis–trans*-isomerisation.

(3) The equilibria are, in most cases, fairly rapidly established.

(4) It has proved possible to study a wider range of ligands than in simpler cobalt(III) complexes.

Particular attention has been paid to complexes containing carbon ligands. They provide a useful, graded series of ligands, are fairly stable to acid, alkali, and cyanide, and give many equilibrium constants within an easily measurable range. Additional interest in these organometallic complexes is provided by the fact that the naturally-occurring coenzymes are also alkyl complexes.

Before commencing the study of the equilibrium it is necessary to establish the structures of the molecules and the valency of the cobalt. Knowledge of the structures depends ultimately on the *X*-ray analysis of, for example, B_{12}[2] and DBC.[3] * Both of these are

* DBC is α-(5,6-dimethylbenziminazolyl)cobinamide coenzyme.

[1] Part III, H. A. O. Hill, J. M. Pratt, and R. J. P. Williams, *J.*, 1965, 2859.

[2] D. C. Hodgkin, *Fortschr. Chem. org. Naturstoffe*, 1958, **15**, 167.

[3] P. G. Lenhert and D. C. Hodgkin, in "Vitamin B_{12} and Intrinsic Factor," ed. Heinrich, Ferdinand Enke, Stuttgart, 1962, p. 105.

diamagnetic cobalt(III) complexes.[4] Other compounds have been related to them either by the stoicheiometry of a reaction or of an equilibrium (*e.g.*, dicyanocobalamin [5]) or by the method of preparation (*e.g.*, methylcobalamin [6,7]) and similar methods have been used in the present work. In addition we have determined the oxidation number of the cobalt and the charge on the complex (and hence the nature of the ligand) of ethynyl- and vinylcobalamin by e.s.r. and electrophoresis, respectively. Octahedral co-ordination, where one of the ligands is a solvent molecule (H_2O), rather than square-pyramidal configuration is difficult to establish conclusively. But in view of the known strong preference of cobalt(III) for octahedral co-ordination and the parallel in spectra between "aquo"-corrinoids and others known to possess six ligands,[8] it will be assumed that all the complexes to be discussed in this Paper are octahedral. A subsidiary uncertainty regarding these complexes is the orientation of the two axial ligands. Because of the relative orientation of the side-chains and the slight deviation from planarity of the corrin ring the equatorial ligand has an "upper" and a "lower" side and two isomers are theoretically possible when the axial ligands are not identical. The benziminazole occupies the same co-ordination site in both vitamin B_{12} and DBC, namely on that side of the ring towards which the nucleotide side-chain projects; the same orientation is assumed for the other cobalamins. No *X*-ray analysis has yet been reported of a cobinamide, but in Factor V_{1a} the cyanide is on the side opposite to that in vitamin B_{12}.[9] No assumptions can therefore be made about the configuration of cobinamides containing two different axial ligands. However, as shown in the Experimental section, the spectrum of methylcobinamide is identical within experimental error with that of the corresponding cobalamin in strong acid, where the base becomes protonated and replaced by H_2O as ligand. This means either that the configuration round the cobalt is the same in the cobalamin and cobinamide or that a change in configuration has no observable effect on the corrin ring, in which case it is reasonable to assume that the equilibrium constant for ligand exchange will be virtually unaffected by a change in configuration. The possibility of co-ordination isomerism will therefore be neglected in discussing the results.

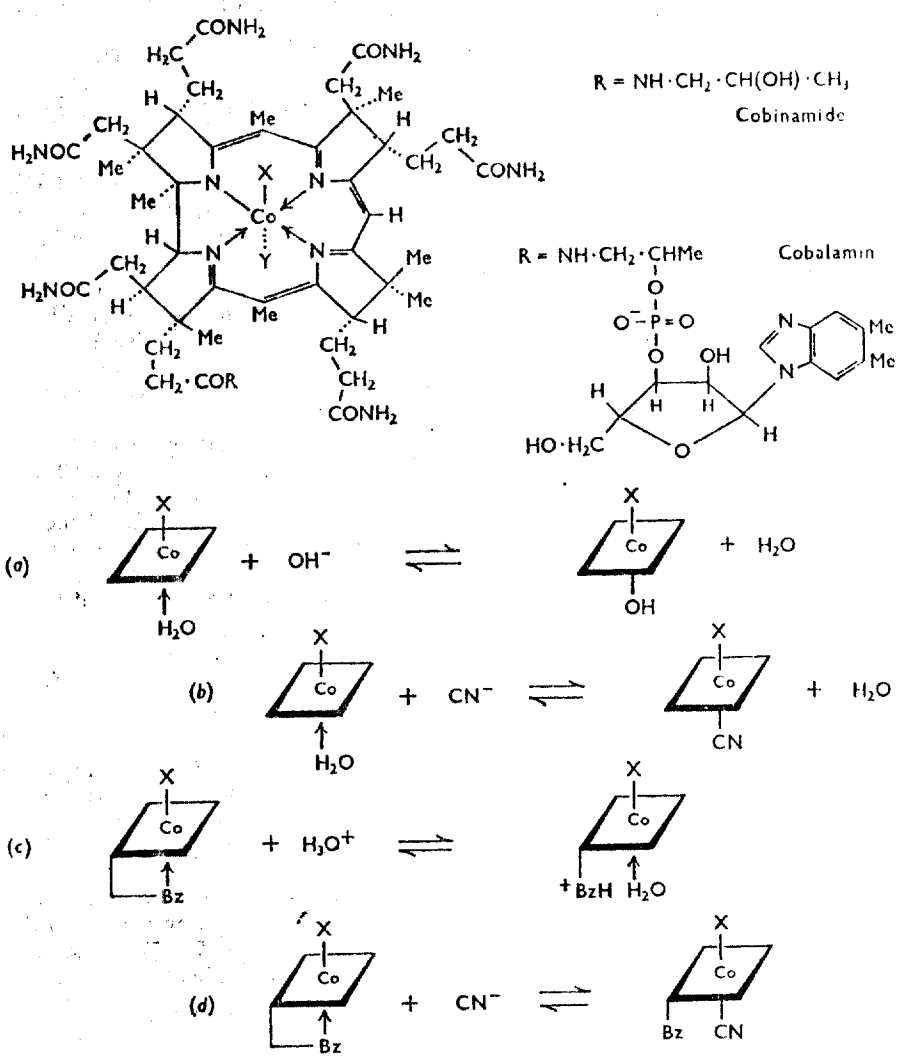
Results

Characterisation of the Compounds.—(*a*) *Vinyl- and ethynylcobalamins.* Although the method of preparation of these cobalamins [7] suggests their formulation as cobalt(III) complexes containing the groups $C_2H_3^-$ and C_2H^- respectively, this has not been proved. We have therefore sought evidence for the presence of unpaired electrons in the compounds and have determined the charge carried by the compounds. Solutions (0·004M) of the vinyl- and ethynyl-cobalamins in water when cooled to 77° K showed no detectable e.s.r. signal, *i.e.*, any signal present was less than 1/400 of that of a corresponding solution of the cobalt(II) derivative, vitamin B_{12r}. Electrophoresis in acetate buffer, pH 5·0, using cyanocobalamin and aquocobalamin as reference compounds (uncharged and one positive charge, respectively) showed that both compounds were uncharged; the spectra in acetate buffer were the same as in water and showed no change over a period longer than the electrophoresis experiment. It is concluded that these compounds, like DBC, can be regarded as diamagnetic cobalt(III) complexes in which the ligand is present as a carbanion ($HC{\equiv}C^-$ and $H_2C{=}CH^-$, respectively) and not as the uncharged hydrocarbon (π-complexes of C_2H_2 and C_2H_4).

(*b*) *Cobinamides.* There are six possible cobinamides with CN^-, H_2O, and OH^- as the only axial ligands. The identity of dicyanocobinamide has been established; it has the same spectrum (λ_γ = 367 mμ) as dicyanocobalamin [5,10] and is uncharged.[10]

[4] R. Bonnett, *Chem. Rev.*, 1963, **63**, 573.
[5] P. George, D. H. Irvine, and S. C. Glauser, *Ann. New York Acad. Sci.*, 1960, **88**, 393.
[6] O. Müller and G. Müller, *Biochem. Z.*, 1962, **336**, 299.
[7] A. W. Johnson, L. Mervyn, N. Shaw, and E. L. Smith, *J.*, 1963, **4146**.
[8] H. A. O. Hill, J. M. Pratt, and R. J. P. Williams, *Proc. Roy. Soc.*, 1965, in the press.
[9] K. Venkatesan, D. H. Dale, and D. C. Hodgkin, Paper presented at the Brussels International Symposium on Organic Chemistry, 1962.
[10] J. B. Armitage, J. R. Cannon, A. W. Johnson, L. F. J. Parker, E. Lester Smith, W. H. Stafford, and A. R. Todd, *J.*, 1953, 3849.

There is some discrepancy in the literature regarding Factor B, which can be formed from the dicyanocobinamide by acidification. Armitage *et al.*[10] concluded from the i.r. spectrum that this compound (λ_γ = 354 mμ) contained no cyanide. Bernhauer, Renz, and Wagner,[11] however, concluded from the infrared spectrum that cyanide was indeed present, and pointed out that the spectrum was identical with that of Factor V_{1a} (λ_γ = 353 mμ) which is known to contain one cyanide.[9,12] Our own work (described in the notes to Table 1) shows that the cyanides of dicyanocobinamide are removed in two steps with very different formation constants (see Table 1). The monocyanide shows a spectrum with λ_γ = 353 mμ in agreement with that given by Bernhauer *et al.*[11,12] The electrophoretic behaviour of Factor B in acid shows that it has one positive charge, establishing the complex as cyanoaquobinamide.[11] It undergoes a reversible change, involving one proton, with a pK = 11 to give the cyanohydroxocobinamide.[13]



George *et al.*[5] claim that the cyanide is lost from Factor B on acidification without, however, affecting the spectrum (λ_γ = 355 mμ in both cases). Bernhauer *et al.*,[11] on the other hand,

[11] K. Bernhauer, P. Renz, and F. Wagner, *Biochem. Z.*, 1962, **335**, 443.
[12] K. Bernhauer, F. Wagner, and D. Wahl, *Biochem. Z.*, 1961, **334**, 279.
[13] B. H. Offenhartz and P. George, *Biochemistry*, 1963, **2**, 142.

claim that the cyanide is not displaced by acid even in light, and give $\lambda_\gamma = 348$ mμ for the cyanide-free complex, which they state without evidence to be diaquocobinamide. We found that a 0·0001M-solution of cyanoaquocobinamide in 0·001N-sulphuric acid showed no change when kept in the dark for several days. On irradiation, however, the γ-band moves from 353 to 348 mμ. If the solution is contained in a sealed cell to prevent the loss of HCN the reaction is slowly reversed on standing in the dark. If the HCN is removed by a stream of air, no regeneration occurs in the dark until a small quantity of KCN is added. The product of photolysis has p*K*s at 6 and ~10·5 (Table 1). The spectrum of the compound present below pH 6 is identical with that of aquocobalamin in strong acid, where the base is protonated and replaced by water, and it is therefore identified as diaquocobinamide. Loss of one and two protons gives aquohydroxo- and dihydroxo-cobinamide, respectively.

Equilibria in Aqueous Solution.—The main equilibria investigated were:

Cobinamides:	replacement of H_2O by OH^- (*a* in Figure)	
	,, H_2O by CN^- (*b* in Figure)	
Cobalamins:	,, benziminazole by H_2O (with protonation of base) (*c* in Figure)	
	,, benziminazole by CN^- (*d* in Figure)	

The equilibria were all studied spectrophotometrically and therefore reflect only those changes directly involving the chromophore (*i.e.*, changes in the conjugated ring itself and in the co-ordination of the cobalt ion) and not changes occurring at the periphery of the molecule (*e.g.*, the protonation of adenine or phosphate in DBC [14] or the possible hydrolysis of the amide side-chains).[10,15] The natures of the complexes were established by the stoicheiometry of the equilibrium together with certain features of the spectra, *e.g.*, comparison of the spectra of the cobalamins and cobinamides above 300 mμ to show whether the co-ordination around the cobalt was the same, and the shift of the benziminazole band from 288 to 285 mμ when protonated.[16,17] In all cases except where otherwise stated the stoicheiometry of the reaction was 1·0 ± 0·02.

The values for the *stoicheiometric* equilibrium constants, *K*, for reactions (*b*) and (*d*) are listed in Table 1. The constants were calculated either directly from the known total free ligand concentration when this was in large excess, or from the known concentrations of reactant and product complexes (known from the spectrophotometric determinations) and the deduced concentration of free ligand, when the total complex and free ligand concentrations were comparable. The free ligand concentration is, of course, the total ligand concentration less the bound ligand. The equilibrium constants for reactions (*a*) and (*c*) are expressed in Table 1 as the acid dissociation constants, pK_a, for the reactions

$$Co(H_2O) \rightleftharpoons Co(OH^-) + H^+$$

and

$$\{Co(H_2O) + BzH^+\} \rightleftharpoons Co(Bz) + H_3O^+$$

respectively, as this is conventional in the literature. The logarithms of the stability constants for the replacement of water by hydroxide (*a*) are the differences between pK_w and pK_a where K_w is the hydrolysis constant. The logarithms of the stability constants for the replacement of benziminazole by water (*c*), at the same time protonating the benziminazole, are identical with the pK_a values given in the Table.

Reversible changes with $pK > 0$ are also shown by solutions of aquocobalamin in the presence of sulphur-containing ligands such as sulphite, thiosulphate, sulphide, cysteine, and thioglycollate. The p*K* in 0·02M-sodium sulphite has already been given as 2·0.[18] In each case the acid solution is yellow and the benziminazole band occurs at 284 mμ, whilst the neutral solution is red, the band occurring at 288 mμ.

Both cobalamins and cobinamides show the presence of one or more additional equilibria

[14] J. N. Ladd, H. P. C. Hogenkamp, and H. A. Barker, *J. Biol. Chem.*, 1961, **236**, 2114.
[15] R. Bonnett, J. R. Cannon, A. W. Johnson, and A. Todd, *J.*, 1957, 1148.
[16] G. R. Beaven, E. R. Holiday, E. A. Johnson, B. Ellis, P. Mamalis, V. Petrow, and B. Sturgeon, *J. Pharm. Pharmacol.*, 1949, **1**, 957.
[17] G. H. Beaven, E. R. Holiday, E. A. Johnson, B. Ellis, and V. Petrow, *J. Pharm. Pharmacol.*, 1950, **2**, 944.
[18] J. A. Hill, J. M. Pratt, and R. J. P. Williams, *J. Theor. Biol.*, 1962, **3**, 423.

in strong acid (conc. H_2SO_4 or $HClO_4$). The number and nature of these complexes has not yet been established, but the previous conclusion [18] that the benziminazole remains bound to the cobalt in the cobalamins has now been shown to be incorrect; in concentrated sulphuric acid the cobalamins and cobinamides have identical spectra above 300 mμ, and the nucleotide shows the same band at 288 mμ whether free or bound in a cobalamin.

TABLE 1

Equilibrium constant measurements

	System	Method	Reagents	pK_a	Note	Ref.
$H_2O \rightarrow OH^-$	Aquocobalamin	1A	0·05M-Phosphate buffers	7·8 (6·9, 7·5, 7·65)	(a)	19–21
	Diaquocobinamide	2A 2B	0·001M-H_2SO_4 + NaOH	6·0 6·1	(b)	
	Cyanocobinamide	2	,,	11·0	(c)	
			Ionic strength → 0	(11·0)		13
	Methylcobinamide	1	Strong NaOH	≥14	(d)	
			?	(>12)		6
				log K		
$H_2O \rightarrow CN^-$	Diaquocobinamide	3C	0·001M-H_2SO_4	≥14	(e)	
	Aquocobalamin	3C	0·2N-Sodium acetate + 0·08N-HCl pH = 4·75	≥12	(e)	
	Cyanocobinamide		?	(8)		5
	Methylcobinamide	2	1N-NaOH + 1N-KCN	3·0 ± 0·2	(f)	
				pK_a		
Benz → H_2O	Aquocobalamin	1B	Conc. H_2SO_4 + H_2O	−2·4 ± 0·2	(g)	
	Cyanocobalamin	1B	,, ,,	+0·1	(h)	
	Ethynylcobalamin	2A	Dilute H_2SO_4	+0·7	(i)	
	Vinylcobalamin	2A	Dilute H_2SO_4	+2·4	(i)	
	Methylcobalamin	1A	0·2N-Acetate/0·08N-HCl buffers	+2·5	(i)	
			?	(+2·7)		22
	DBC	1A	0·2N-Acetate/0·08N-HCl buffers	+3·35	(i)	
			Various	(3·3—3·5, 3·52)		14, 22
				log K		
Benz → CN^-	Cyanocobalamin	?	?	(4)		5
	Ethynylcobalamin	2A	1N-NaOH + KCN	2·7	(j)	
	Vinylcobalamin	1A	,,	0·7	(j)	
	Methylcobalamin	1B	,,	0·1	(j)	

An estimated error of ±0·1 applies to all log (equilibrium constants) except where otherwise stated.

Notes on Table 1. (*a*) There is no spectrophotometric evidence for the formation of a phosphate complex under these conditions. The difference between our value and that of Hanania and Irvine (7·65),[21] the most reliable in the literature, is within experimental error.

(*b*) Diaquocobinamide was prepared by photolysing a 0·0001—0·001M-solution of Factor B in $HClO_4$ or H_2SO_4, pH 2—4, until no further change in spectrum was observed, the HCN being removed by a stream of air passing through the solution. The resulting solution showed the same spectrum above 300 mμ as vitamin B_{12a} in strong acid [see under (*h*)]:

	λ_γ	λ_β	λ_α	ε_{molar}
Diaquocobinamide	348	490	519	$2·8 \times 10^4$
B_{12a} in strong acid	348	491	518	$2·8 \times 10^4$

The constants were determined in perchloric or sulphuric (~0·001M) rather than in hyrochloric acid because of the formation of chloride complexes. In a separate experiment in strong acid

[18] R. P. Birks, E. G. Newstead, and N. R. Trenner, *Science*, 1951, **113**, 625.
[20] E. L. Smith K. H. Fantes, S. Ball, J. G. Waller, M. B. Emery, W. K. Anslow, and A. D. Walker, *Biochem. J.*, 1952, **52**, 389.
[21] G. I. H. Hanania and D. H. Irvine, Paper presented at the 8th International Conference on Co-ordination Chemistry, Vienna, 1964.
[22] D. Dolphin, A. W. Johnson, and R. Rodrigo, *Ann. New York Acad. Sci.*, 1964, **112**, 590.

solutions (2N-H_2SO_4) the stability constant of the chloride complex of diaquocobinamide has been shown to be 3·3 ± 0·2. Difficulties were also experienced owing to the sulphur dioxide in the atmosphere. Solutions of diaquocobinamide at very low or high pH show little or no change in spectrum over long periods, but over the range pH 5—11 the solutions gradually become yellow. The position of the isosbestic points during this reaction and the spectrum and light-sensitivity of the product identified it as sulphitocobinamide, and the source of contamination was shown to be the atmosphere. In 0·05M-sodium borate the log (stability constant) of the sulphite complex of aquohydroxocobinamide, which is formed rapidly, exceeds 8. Despite these experimental problems the agreement between the two methods of determining the constant is good.

(*c*) This equilibrium was studied to check the value given by Offenhartz and George.[13] Armitage *et al.*[10] had reported a p*K* of 6·8 for Factor B, but it is not clear from their Paper whether they were studying the diaquo- or the monocyano-cobinamide, though Bonnett[4] assumes it was the latter. Our value agrees with that of Offenhartz and George.[13] Factor B, like diaquocobinamide, reacts with sulphite to give sulphitocobinamide, but the reaction is slower and far less serious. This may have been the reaction observed by Offenhartz and George[13] and attributed by them to undefined "aggregation phenomena."

(*d*) No change in spectrum was observed between 1N-sulphuric acid and 10N-potassium hydroxide. Thus, the ionisation of the bound H_2O to OH^- does not occur in an observable pH range. The appearance of the band at 285 mμ indicates that the benziminazole is protonated in the acid form.[16,17]

(*e*) Vitamin B_{12} and Factor B were photolysed until complete conversion into B_{12a} and diaquocobinamide had occurred, and then left in the dark at room temperature to allow reformation of the cyanide complex. The experimental data were as follows:

	B_{12}	Factor B
Concentration	$2·5 \times 10^{-5}$M	$2·9 \times 10^{-5}$M
Solution	Acetate buffer	0·01N-H_2SO_4
pH	4·75	2·0
$t_{\frac{1}{2}}$ of dark reaction	4½ hr.	~2 days
% reformation (time)	>98% (after 6 days)	>93% (after 17 days)

The minimum constants given in Table 1 were calculated on the basis of the p*K* of HCN being 9·3.[23] George *et al.*[5] also give figures for these equilibrium constants, but the calculations were based on wrong assumptions as to the nature of the equilibria observed [see under (*h*)].

(*f*) Slight decomposition of the methylcyanocobinamide was noted at high cyanide concentrations, which may have been due to the extreme light-sensitivity of the complex. A weak complex between methylcobinamide and cyanide has already been noted by Müller and Müller.[6] The result quoted is the mean of three determinations.

(*g*) The acid end-point cannot be observed because of overlap with a further equilibrium (or equilibria) involving the complexes present in concentrated sulphuric acid. The spectrum of the fully-formed complex can, however, be obtained by diluting a solution of vitamin B_{12a} in concentrated acid with water, since the equilibrium involing the reversible displacement of the base is set up relatively slowly in acid. The benziminazole band occurs at 284 mμ, indicating that the base is protonated,[16,17] and the spectrum above 300 mμ is identical with that of the diaquocobinamide [see under (*b*)]. The equilibrium constant was derived using the Hammett acidity function H_0.[24]

(*h*) A further equilibrium also obscures the acid end-point in the case of vitamin B_{12}. Thus again method 1B was used and the equilibrium constant derived using the Hammett acidity function H_0. Beaven *et al.*[17] observed a shift in the γ-band of B_{12} to shorter wavelength at pH −1 and concluded that the base was reversibly displaced and protonated with p*K* ~0. George *et al.*[5] apparently observed the same equilibrium but because their experimental data could not be fitted to a single equilibrium they concluded that both the cyanide and the benziminazole were displaced. They used hydrochloric acid solutions, however, and since chloride can act as a ligand (see (*b*)) their results probably reflect the displacement of the base by both Cl^- and H_2O.

(*i*) The acid forms of ethynyl-, vinyl-, and methyl-cobalamin and DBC are all yellow and

[23] *Chem. Soc. Special Publ.* No. 7, Part II, 1958.
[24] M. A. Paul and F. A. Long, *Chem. Rev.*, 1957, **57**, 1.

in each case the benziminazole band occurs at 284 mμ. Acid catalyses the decomposition of ethynyl- to acetyl-cobalamin, identified by comparison of the spectra in acid and alkali with those of a sample of acetylcobalamin ($t_{\frac{1}{2}}$ ~10 min. in 1N-sulphuric acid at room temperature). Acid catalysed the decomposition of vinylcobalamin to vitamin B_{12a} ($t_{\frac{1}{2}}$ ~45 min. in 10N-sulphuric acid at room temperature). In addition a further reaction or equilibrium of vinylcobalamin was observed in strong acid, marked by an increase in absorption around 310 mμ. The acid end-points could not, therefore, be obtained in these two cases. Methylcobalamin, however, is completely stable to acid, and DBC is stable over the range required for determining the pK. The acid form of methylcobalamin has the same spectrum above 300 mμ as methylcobinamide:

		Main bands (mμ)			ε_{303} : ε_{458}
Methylcobinamide in water	--	302·5	372	458	2·40
Methylcobalamin in 1N-H_2SO_4	285	303	372	458	2·35

Ladd *et al.*[14] report that the equilibrium of DBC, as determined by titration and paper iono-phoresis, involves two protons, but conclude that only one proton is involved in the change of spectrum (in agreement with our result), the other being associated with the adenine nucleotide. The buffers used in these determinations do not affect the spectra of the complexes.

(*j*) The equilibrium constants for replacing benziminazole by cyanide in aquocobalamin and DBC cannot be determined. Cyanide decomposes DBC to dicyanocobalamin; excellent isosbestic points were observed during the reaction in 0·1N-potassium cyanide and no evidence was obtained for any intermediate. Vitamin B_{12a} reacts with one mole of cyanide to give vitamin B_{12} and with an excess to give dicyanocobalamin. Ethynyl-, vinyl-, and methyl-cobalamin, however, show a reversible reaction with cyanide. The spectra of methylcobin-amide and methylcobalamin in excess of cyanide appear to be the same above 300 mμ (main bands at 388 and 568 mμ and ~390 and ~565 mμ, respectively). Dolphin *et al.*[22] claim that cyanide decomposes alkylcobalamins to dicyanocobalamin, but do not state which compounds they have studied. The constants were all determined at high sodium hydroxide concentration so as to control ionic strength and pH.

Discussion

The data in Table 2 show, first of all, that very large changes in the equilibrium constants for ligand substitution can be brought about simply by changing the *trans*-ligand (a variation of over 10^{11} for the replacement of H_2O by CN^-); secondly, that the stability constant order for the ligands is the same for each type of equilibrium (*a*), (*b*), and (*d*) but is reversed for (*c*) where the entering ligand is the weaker donor; and, thirdly, that as the fixed ligand is changed in the order H_2O, NC^-, $HC{\equiv}C^-$, $H_2C{=}CH^-$, CH_3^- (and then C'_5-de-oxyadenosyl in the one equilibrium for which a constant is reported) so the stability of the *trans* cobalt–cyanide bond decreases relative to that of the cobalt–benziminazole bond (*d*) which in turn decreases relative to that of the cobalt–water bond, *i.e.*, $-pK_a$ in (*c*).

Previous measurements[25] on the thermodynamic *trans*-effect using Pt^{II}, Co^{III}, Fe^{II}, and Rh^{III} complexes have shown it to be small but in all these cases closely similar ligands were involved in the equilibria. As a consequence it has proved very difficult to establish the cause of the *trans*-effect. Both the thermodynamic and the kinetic *trans*-effects have been discussed in terms of inductive effects, π-bonding, polarisation, etc. Of the two, the thermodynamic equilibrium constants should be the easier to interpret because they involve only ground states of known stereochemistry. We hope to study further equili-brium constants of the corrinoids with a view to separating out the several possible effects. However, the results obtained so far—the similar effect of carbon and sulphur anions in general and the order cyanide < ethynyl < vinyl < methyl in particular—support the correlation of the *trans*-effect with the ability of the ligand atom to reduce the effective nuclear charge on the cobalt atom (see, for example, Basolo and Pearson[25]). The influence

[25] F. Basolo and R. G. Pearson, *Progr. Inorg. Chem.*, 1962, **4**, 381, Pt^{II} Co^{III}; B. A. Jillot and R. J. P. Williams, *J.* 1958, 462, and R. C. Davies, D.Phil. Thesis (Oxford) 1963, Fe^{II}; E. J. Bounsall and A. J. Poë, Abstracts of The Eighth International Co-ordination Chemistry Conference, Vienna (1964), p. 313, Rh^{III}.

of the carbon ligands on the wavelength of the γ-band in the cobalamins follows the same order and has been correlated with the electronegativity of the carbon atom.[4]

It is interesting to note that the cobalt atom when attached to an alkyl group shows no marked preference for carbon, nitrogen, or oxygen as the sixth ligand atom. Furthermore, all the corrinoids studied are unusual in being kinetically labile (towards the replacement

TABLE 2

Summary of equilibrium constants *

	Cobalamins		Cobinamides	
	(c)	(d)	(a)	(b)
trans-Ligand	Benz ⟶ H_2O log K (= pK_a) *	Benz ⟶ CN^- log K	H_2O ⟶ OH^- pK_a *	H_2O ⟶ CN^- log K
H_2O	−2·4	(⟶ B_{12})	6·0	⩾14
^-CN	+0·1	4	11·0	8
$^-C{:}CH$	+0·7	2·7	—	—
$^-CH{:}CH_2$	+2·4	0·7	—	—
$^-CH_3$	+2·5	0·1	>14 (No pK detected)	3·0
$^-C_5'$-Deoxyadenosyl (DBC)	3·4	(⟶ dicyanide)	—	—

* See the Figure for the chemical formulation of the equilibria. The stability constant for reaction (a) is $pK_w - pK_a$ and for reaction (c) is equal to pK_a, see p. 6488.

of at least one axial ligand), in contrast with the inertness of most cobaltic complexes.[26] Both thermodynamically and kinetically, therefore, the cobalt atom of the coenzymes could be reactive towards a wide range of compounds *in vivo*.

EXPERIMENTAL

Reagents.—Samples of the corrinoids were given by Dr. E. Lester Smith, F.R.S., and Professor D. C. Hodgkin, F.R.S. With the exception of Factor B the samples were used without further purification. The samples of Factor B contained an excess of cyanide which was easily removed by passing a stream of air through the solution in the dark at pH 3 to leave the pure monocyanide (see under Results). AnalaR reagents were used wherever possible. [It should be noted that two of the compounds are unstable to strong acid. Acid catalyses the conversion of ethynyl- into acetyl-cobalamin, recognised by its spectrum. Compare the analogous hydration of acetylenes to aldehydes, catalysed by mercury(II) salts. Vinylcobalamin decomposes to aquocobalamin, perhaps *via* the addition of a proton to give the π-olefin complex.]

Absorption Spectra.—The spectra were taken with a Beckmann DK ratio-recording spectrophotometer and also with a Unicam S.P. 600, using 1 cm. silica cells. Molar extinction coefficients were determined by conversion into the dicyanide using the molar extinction coefficient[4] of λ_γ of the dicyanide = $3{\cdot}04 \times 10^4$.

Photolysis.—The solutions to be photolysed were contained in a sealed cell and placed 10—25 cm. from a 230 v, 125 w Osram mercury lamp.

Measurement of pH.—Most pH measurements were made on small (~2 ml.) quantities of solution in a spectrophotometer cell. We used either a Radiometer or Cambridge pH meter in conjunction with either a Radiometer GK 264 combined electrode which fitted into the B10 socket of a cell, or very slim glass and calomel electrodes which fitted into a straight-sided cell.

Electron Spin Resonance.—The samples, cooled in liquid nitrogen, were examined in a Varian X-band spectrometer, V 4500, in a double-cavity cell, using a Mn^{2+} in MgO sample as reference; the spectrum was run from 0·5 to 4 kilogauss with a scan rate of 350 gauss per inch; the modulation width was 0·5 gauss at 100 kc./sec. No signal was detected from solutions of vinyl- or ethynyl-cobalamins, under the same conditions solutions containing the same concentration of vitamin B_{12r} exhibited prominent signals.[27]

Electrophoresis.—Experiments were carried out in a modified B.T.L. C6/5400 horizontal electrophoresis tank at 200 v. The following Table gives the conditions and rates of migration

[26] D. R. Stranks, "The Reaction Rates of Transition Metal Complexes" in "Modern Co-ordination Chemistry," ed. J. Lewis and R. G. Wilkins, Interscience, New York, 1960, p. 78.

[27] H. P. C. Hogenkamp, H. A. Barker, and H. S. Mason, *Arch. Biochem. Biophys.*, 1963, **100**, 353.

of the different compounds. The charge on the vinyl and ethynyl compounds are obviously the same as that on B_{12} at all the recorded pH values.

Supporting electrolyte	Volts	Time (hr.)	Movement (cm.) B_{12}	B_{12a}	Vinyl	Ethynyl
0·01M-NaOH	200	2	1·8	1·8	1·8	1·8
Acetate/HCl buffer pH 5·0	200	2½	0·8	−0·2	0·8	0·7
0·02N-CH_3CO_2H	400	2½	−2·7	−9·0	−2·9	

Determination of Equilibrium Constants.—All equilibrium constants were determined spectrophotometrically. Three different methods of adding the reagent (1—3) and three different methods of analysing the results were used (A—C).

(1) A range of solutions of varying reagent concentration was used.

(2) The corrinoid solution in a 1-cm. cell was titrated with a solution of the reagent (a series of increasingly concentrated solutions was often used) from a micrometer syringe fitted with a hypodermic needle.

(3) In order to obtain an estimate of the equilibria between cyanide and aquocobalamin and diaquocobinamide, which have high formation constants ($\geqslant 10^{12}$), a known, very low concentration of cyanide was generated *in situ* by the complete photolysis of a solution of the cyano-complex in acid inside a sealed spectrophotometer cell to prevent loss of HCN. The concentration of cyanide ion was calculated from the pK of HCN, the pH of the solution and the concentration of the corrinoid.

All the equilibria studied involved the substitution, by a reagent (Y) such as CN^-, of either H_2O in the cobinamides or of the nucleotide benziminazole in the cobalamins; the concentration of water or benziminazole can therefore be neglected in the equilibrium $A + nY \rightleftharpoons B$, where A and B are both corrinoids. The experimental parameters were: the optical density at the given wavelength corresponding to 100% A (ε_A), 100% B (ε_B), or intermediate values (ε_Y) in equilibrium with a known concentration of the reagent, Y.

$$\therefore \quad K = \frac{[B]}{[A][Y]^n} = \frac{\varepsilon_Y - \varepsilon_A}{(\varepsilon_Y - \varepsilon_B)[Y]^n}$$

(A) Where ε_B was experimentally observable the experimental data were plotted as log [Y] against log [B]/[A]. Unit slope proved the participation of one mole of reagent in the equilibrium; $K = 1/[Y]$ when log [B]/[A] = 0.

(B) When ε_B was not experimentally observable because of a second overlapping equilibrium, irreversible decomposition at high concentrations of the reagent, or a very low formation constan the data were analysed by the following method.[28,29] From the above equation

$$(\varepsilon_B - \varepsilon_Y) = \frac{\varepsilon_A - \varepsilon_Y}{K[Y]^n} \text{ or } -\varepsilon_Y = \frac{\varepsilon_A - \varepsilon_Y}{K[Y]^n} - \varepsilon_B$$

A plot of ε_Y against $(\varepsilon_A - \varepsilon_Y)/[Y]$ yields a straight line if $n = 1$, the slope of which is equal to $1/K$ and the intercept to ε_B. The experimental results could be re-evaluated by the first method using a value of ε_B obtained either by this second method (referred to in Table 1 as method A_B) or, where deviation occurs only over the last 5% or so of the equilibrium, by first determining an approximate equilibrium constant by the first method (A_A).

(C) Minimum values only for the equilibrium constant between CN^- and aquocobalamin and diaquocobinamide were obtained from the degree of reformation of the cyano-complex observed after standing in the dark.

In all cases, values of [Y] were corrected for reagent bound by the complex, and values of ε for dilution if necessary.

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[28] A. K. Lunn and R. A. Morton, *Analyst*, 1952, 77, 718.
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ABSORPTION OF VITAMIN B_{12} FROM THE COLON OF THE PIG [1, 2]

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THE intestinal flora of monogastric animals is capable of producing vitamin B_{12} (Dyke and Hind, 1950; Davis and Chow, 1951; Johansson *et al.*, 1953). Since the major part of the intestinal flora is in the lower digestive tract, it is important to determine the rate at which vitamin B_{12} is absorbed or metabolized in this region.

Citrin *et al.* (1957) stated that a very small portion of large doses (500 to 2000 mcg.) of vitamin B_{12} was absorbed, presumably by diffusion through most mucosal surfaces. In experiments with humans the same authors could not demonstrate any absorption of 0.5 mcg. of Co^{60} labeled vitamin B_{12} from the colon. Using doses of 100 mcg. vitamin B_{12}, Monto and Rebuck (1955) demonstrated absorption from the nasal mucosa. Ross *et al.* (1954) and Reisner *et al.* (1955) reported that some absorption took place following rectal administration of 500 mcg. or more of vitamin B_{12} in humans.

The experiments reported herein were conducted to determine the rate of absorption of vitamin B_{12} from the colon of the pig, using a colon cannula and Co^{57} labeled vitamin B_{12}.

Experimental Procedure

Using the technique described by Redman *et al.* (1964), in each of two experiments, three Duroc barrows, weighing between 17 and 26 lb., were provided with a cannula inserted into the ascending colon. Four weeks postoperative the pigs were placed in metabolism cages. Feces were collected in plastic bags attached to a metabolism harness and the urine drained into containers below the cages. The technique allowed a complete separation of feces and urine.

Following a 6-day preliminary period in the cages, 2 ml. of a radioactive vitamin B_{12} solution was introduced through the cannula into each pig. The 2 ml. of solution contained a total of 0.114 mcg. of vitamin B_{12} with a specific activity of 4.48 microcuries per microgram. Thus, 0.5 microcuries of Co^{57} labeled vitamin B_{12} were introduced. Distilled water was used to rinse the cannula following the introduction of the radioactive vitamin B_{12} solution.

While in the metabolism cages, the pigs had free access to water and feed. The ration had the following percent composition: ground shelled corn, 78.62; meat and bone scrap, 6.40; soybean oil meal, 12.70; steamed bone meal, 1.00; trace mineralized salt, 0.40; and vitamin-antibiotic-zinc premix, 0.88.

The collection period lasted for 7 days, after which time the animals were slaughtered. In the second experiment the heart, kidney, liver and spleen were obtained for tissue analysis.

Radioactivity was measured with an all sodium iodide, thallium activitated crystal in a well-type scintillation counter coupled to a scaling unit. Activity in the urine was counted after filtration through Whatman No. 1 filter paper. The feces were dried in an air draft oven at 55° C., ground and extracted according to the A.O.A.C. (1960) method for vitamin B_{12} analysis. After centrifugation the extract was adjusted to volume and counted. A urine and feces sample obtained during the preliminary feeding period was used for the background correction.

To obtain a measure of vitamin B_{12} in the fecal samples, daily extracts which possessed the highest radioactivity were treated according to Johnson *et al.* (1956) with activated charcoal (Nuchar C-190-N) and the suspension filtered through a Buchner funnel. The filtrate was then adjusted to volume and counted.

For determining radioactivity in organ tissue, the tissue was homogenized and extracted. Radioactivity was counted in the same manner as for feces and urine samples.

Results and Discussion

The percent of radioactivity recovered in the excreta is shown in table 1. The highest

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TABLE 1. PERCENT RECOVERY OF Co^{57} IN EXCRETA

Amount introduced	Experiment 1: 0.51 μc. in 0.114 mcg. vitamin B_{12}			Experiment 2: 0.52 μc. in 0.114 mcg. vitamin B_{12}		
Pig	A	B	C	D	E	F
			Feces			
Day 1	16.7	0.5	12.9	5.2	0.4	18.5
2	33.8	3.8	32.1	30.6	32.6	22.6
3	1.9	30.1	6.1	4.4	12.0	4.2
4	1.4	12.5	4.9	0.6	5.0	1.1
5	0.1	0.5	0.4	1.1	1.1	0
6	0	1.8	1.2	0.1	1.4	0.4
7	0.1	1.1	0.8	0	0.9	0.5
Total in 7-day period	54.0	50.3	58.4	42.0	53.4	47.3
			Urine			
Total in 7-day period	1.9	1.9	2.0	2.5	2.5	1.6
Total excreted	55.9	52.2	60.4	44.5	55.9	48.9

amount of radioactivity appeared in the second 24-hr. collection of feces with the exception of pig B. In this animal the greatest amount appeared in the third collection period. Variation from animal to animal was apparently associated with rate of passage of ingesta through the large intestine and time of defecation in relation to collection. During the last 3 days of the balance trials, only a small amount of Co^{57} appeared in the feces with the maximum during this period being 3.5% of the injected dose. Thus, a major portion of the introduced quantity of B_{12} was exposed to only 4 days of absorption or bacterial alteration in the colon.

For all six pigs an average of 50.8% of the introduced radioactivity was excreted in the feces during the experimental period, leaving 49.2% apparently absorbed. During the same period there was an average of 2.1% excreted in the urine; thus, 47.1% of the injected radioactivity was retained in the body of the pig.

At the time of slaughter the cannulas were removed and checked for radioactivity. None was detected. Radioactivity in organs removed from the three barrows used in Experiment 2 is presented in table 2. The quantities are listed as a percent of the total dosage introduced into the colon. It can be seen that all organs contained some radioactivity. The highest amount was found in the liver, averaging 14.4% of the original dose. Variation among animals was small, and the average recovery in the organs examined was 21.4%. This leaves 25.7% of non-recovered radioactivity which was apparently in other body tissues. The presence of radioactivity in these four organs and in the urine is evidence that Co^{57} was absorbed from the large intestine.

TABLE 2. PERCENT RECOVERY OF Co^{57} IN ORGAN TISSUE SEVEN DAYS AFTER INTRODUCTION INTO COLON (EXPERIMENT 2)

Tissue	Pigs D	E	F
Heart	4.4	3.0	3.2
Kidneys	2.7	2.4	2.3
Liver	14.8	13.5	14.9
Spleen	0.9	1.1	0.9
Total	22.8	20.0	21.3

The measured radioactivity in the organs was, however, not necessarily vitamin B_{12}. It is possible that vitamin B_{12} was broken down by bacterial action and that cobalt in ionic form or as some portion of the molecule was set free and absorbed. To determine whether or not the radioactivity in the fecal samples was actually B_{12}, the two feces extracts with highest radioactivity were treated with charcoal in order to adsorb the vitamin B_{12}. These results are presented in table 3. While only 0.1% of the radioactivity in the injected solution was not adsorbed, an average of 18.9% of the activity in the feces samples was not adsorbed on the charcoal. Thus, it is apparent that during passage through the large intestine a portion of the vitamin B_{12} was modified, probably as a result of being metabolized by microbial action. The metabolism of vitamin B_{12} by microorganisms is not well known nor is the stage at which metabolized vitamin B_{12} does not adsorb on charcoal. It does, however, appear safe to conclude that only the Co^{57} ad-

TABLE 3. PERCENT ADSORPTION OF FECAL RADIOACTIVITY ON CHARCOAL

Item	Solution introduced into colon	Experiment 1			Solution introduced into colon	Experiment 2		
		Extract of feces from pig				Extract of feces from pig		
		A	B	C		D	E	F
Pooled fecal sample of day	....	1 + 2	3 + 4	1 + 2	....	2 + 3	2 + 3	1 + 2
Adsorbed, %	99.9	79.8	82.3	81.3	99.9	79.7	85.6	78.0

sorbed on charcoal possessed vitamin B_{12} activity. Since 99.9% of the radioactivity in the injected material and only 81.1% of that in the feces was adsorbed on charcoal, it is evident that at any place between the site of introduction into the colon and the anus true vitamin B_{12} constituted at least 81.1% of the radioactivity. This would also be the apparent quantity of radioactive B_{12} available for absorption.

If vitamin B_{12} absorbed from the large intestine contributes to the animal's requirements, the amount absorbed would be dependent on different factors. Among these would be the amount of dietary vitamin B_{12} reaching the colon in the free state and the amount of vitamin B_{12} synthesized by bacteria and liberated from the microbial cell in this part of the tract, as well as the unknown factors related to passage of B_{12} through the intestinal mucosa. Since 49.2% of the injected radioactive vitamin B_{12} did not pass out in the fecal material, this would mean that 0.054 micrograms could have been absorbed. Because of the vitamin B_{12} content of the feed and possible production of vitamin B_{12} by the flora in this region of the tract, the total amount absorbed during this period may have been higher. Dietary vitamin B_{12} which escaped absorption before reaching the site of the cannula would, in terms of the radioactivity available for absorption, further dilute the labeled vitamin.

Summary

Six pigs were provided with colon cannulas introduced into the first portion of the colon via the cecum. Vitamin B_{12} labeled with Co^{57} was introduced into the colon. By the collection of feces and urine during a 7-day period, together with analysis of organ tissue, it was demonstrated that 41.6 to 58.0% of the radioactive cobalt was absorbed. Based upon the charcoal adsorption of radioactivity present in the feces, it appeared that some vitamin B_{12} was metabolized during its passage through the lower digestive tract.

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Vitamin B_{12} and protein metabolism

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In the last few years numerous papers have linked vitamin B_{12} in a general way with the metabolism of proteins in different animals.

We reported some years ago (Henry & Kon, 1951) that addition of vitamin B_{12} increased the biological value of casein for the vitamin B_{12}-deficient rat. Indian authors (Marfatia & Sreenivasan, 1951; Baliga & Rajagopalan, 1954; Baliga, Balakrishnan & Rajagopalan, 1954) obtained similar increases for vegetable proteins with normal rats. There was, however, a growing indication of a more specific connexion between vitamin B_{12} and biosynthesis of the essential amino-acid methionine (see, for example, Schaeffer, Salmon & Strength, 1949; Oginsky, 1950) through its effect on the formation of methyl groups (see review by Arnstein, 1955). We have since investigated the bearing of these findings on our original observation, and the results are set out below.

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EXPERIMENTAL

Plan of the experiments

Three groups of experiments were done and their general plan is summarized in Table 1.

Table 1. *Protein source and amount of protein in, and nitrogen content of, the experimental diets*

Exp. no.	Protein source: Description	Amount in diet (%)	N in diet (%)
	Experiments with vitamin B_{12}-deficient rats		
1	Casein*	9·0	1·212
2	Casein*	8·5	1·234
	Casein + 0·5 % DL-methionine*	8·5	1·235
3	Casein*	8·5	1·181
	Casein + 0·5 % DL-methionine*	8·5	1·181
5	Casein	8·6	1·175
	Casein + 1 % DL-methionine	8·6	1·146
	Casein + 1 % homocysteine + 0·5 % choline†	8·6	1·178
6	Casein	8·6	1·172
	Casein + 1 % DL-methionine	8·6	1·173
	Casein + 1 % homocysteine + 0·5 % choline	8·6	1·181
8	Casein	8·6	1·177
	Casein + 1 % DL-methionine	8·6	1·159
	Casein + 1 % homocysteine*	8·6	1·170
10	100 % extraction wheat flour*	63·7	1·398
	100 % extraction wheat flour + 0·5 % DL-methionine	63·7	1·395
11	100 % extraction wheat flour*	63·7	1·398
	100 % extraction wheat flour + 0·5 % DL-methionine	63·7	1·395
	100 % extraction wheat flour + 1 % L-lysine*	60·0	1·404
	100 % extraction wheat flour + 1 % L-lysine + 0·5 % DL-methionine*	60·0	1·393
	Experiments with normal rats		
4	Casein*	8·5	1·201
	Casein + 0·5 % DL-methionine*	8·5	1·202
7	Casein	8·6	1·211
	Casein + 1 % DL-methionine	8·6	1·121
	Casein + 1 % homocysteine + 0·5 % choline*	8·6	1·190
9	Casein	8·6	1·201
	Casein + 1 % DL-methionine	8·6	1·189
	Casein + 1 % homocysteine*	8·6	1·192
12	100 % extraction wheat flour*	61·4	1·370
	100 % extraction wheat flour + 0·5 % DL-methionine*	61·4	1·287
	100 % extraction wheat flour + 1 % L-lysine*	58·2	1·364
	100 % extraction wheat flour + 1 % L-lysine + 0·5 % DL-methionine*	58·2	1·274
13	Soya-bean meal*	14·8	1·211
	Soya-bean meal + 1·6 % DL-methionine*	14·8	1·213
14	Casein (8 % protein)*	8·6	1·343
	Casein (16 % protein)*	17·5	2·408

* One group of rats without vitamin B_{12}, another with 0·2 μg vitamin B_{12} daily.
† Rats received 0·2 μg vitamin B_{12} daily.

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Effect of vitamin B_{12} on the biological value of casein

The effect of additions of vitamin B_{12} or methionine or both on the biological value of casein was studied with vitamin B_{12}-deficient (Exps. 1–3) and normal (Exp. 4) rats. Both kinds of rat were used also to test the possibility that vitamin B_{12} is involved in transmethylation processes, and for this purpose the effect of additions of choline and homocysteine to casein with or without vitamin B_{12} was studied (Exps. 5–7). Finally, to test the now accepted view that vitamin B_{12} is involved in the synthesis of methyl groups, the effect of the addition of vitamin B_{12} to casein supplemented with homocysteine was determined with deficient (Exp. 8) and with normal (Exp. 9) rats. In all these experiments protein constituted 8% of the diet.

Effect of vitamin B_{12} on the utilization of vegetable proteins

With deficient rats two experiments were done. In one (Exp. 10) the effect of vitamin B_{12} on the biological value of the proteins of 100% extraction wheat flour was compared with that of added methionine. In the other (Exp. 11) the protein-efficiency ratio of the same flour with the above supplements was determined, and, in addition, the effect of the vitamin on the protein-efficiency ratio of the flour with supplements of lysine or of lysine and methionine. Exp. 11 was repeated on normal rats (Exp. 12); an extra group of rats was included, for which the flour was supplemented simultaneously with methionine and vitamin B_{12}. A second experiment with normal rats (Exp. 13) dealt with the effect of vitamin B_{12} on the biological value of soya protein alone or supplemented with methionine.

In Exps. 10–13 the 8% protein level was again used.

Effect of vitamin B_{12} on the biological value of casein at 8% and at 16% levels of protein intake

Vitamin B_{12} is said to stimulate the growth of rats and mice on high-protein diets (Hartman, Dryden & Cary, 1949*a*, *b*; Bosshardt, Paul & Barnes, 1950). It has long been established that the biological value of proteins decreases as the concentration of protein in the diet increases (Mitchell, 1923–4*a*). The purpose of this experiment (Exp. 14) was to determine with normal rats whether vitamin B_{12} would counteract the depression in biological value associated with higher levels of protein.

Diets

Diets used for depleting rats of vitamin B_{12}

Two vitamin B_{12}-deficient diets were used: diet A was based on that described by Emerson (1949), diet B was diet RB 12S/4 of Cuthbertson & Thornton (1952). Table 2 gives their composition.

Experimental diets

The basal diet used in all experiments had the following percentage composition: rice starch 64, sugar 12, potato starch 10, margarine fat 10, salts (de Loureiro, 1931) 4. The protein source to be tested was added in place of an equal weight of rice starch;

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sugar was omitted from the diets containing flour (Exps. 11 and 12). Table 1 gives the nitrogen content of the diets and the amount of the protein source replacing starch. In the experiments with vitamin B_{12}-deficient rats each animal was given daily, on a separate dish, 0·5 ml. of the mixture of water-soluble vitamins described by Campbell & Kosterlitz (1948). The normal rats were given 0·3 ml. of an aqueous extract of brewer's yeast (Osborne & Wakeman, 1919) equivalent to 0·3 g dried yeast. Vitamins A and D were supplied as two drops daily of cod-liver oil per rat.

Table 2. *Percentage composition of the vitamin B_{12}-deficient diets*

Component	Diet A*	Diet B†
Soya-bean meal	60	—
Soya flour (full fat)	—	72
Glucose	25·5	—
Lactose	—	22
Salts (de Loureiro, 1931)	4	4
Groundnut oil (semi-hardened)	10	—
Vitaminized arachis oil‡	—	2
Dried thyroid	0·5	—
Vitamin additions (mg/kg diet):		
Thiamine	10	30
Riboflavin	20	30
Pyridoxin	10	8
Calcium pantothenate	100	100
Nicotinic acid	100	100
Inositol	50	220
p-Aminobenzoic acid	300	75
Choline hydrochloride	1000	—
Biotin	0·5	0·2
Folic acid	2	1
Menaphthone	142	—

* Based on the diet of Emerson (1949).
† Diet RB 12S/4 of Cuthbertson & Thornton (1952).
‡ Vitamin A 2×10^6 i.u., vitamin D_2 10^5 i.u., α-tocopheryl acetate 14·2 g, menaphthone 100 mg and arachis oil to 1000 g.

In all metabolic experiments with depleted rats the ether-extracted whole egg used in the diet in the standardizing periods (see Mitchell & Carman, 1926) was from eggs of hens deficient in the animal protein factor (Black, Getty, Coates, Harrison & Kon, 1949).

Rats

Hooded Norwegian litter-mate rats of our own breeding were used. In accordance with our usual procedure, females were used in the balance experiments with normal animals. The experiments with vitamin B_{12}-deficient rats and the measurements of the protein-efficiency ratio were done with males.

Experimental procedure

Experiments with normal rats

Exps. 4, 7, 9, 13 *and* 14. The biological value and true digestibility of the test proteins were determined by the method of Mitchell (Mitchell, 1923–4*b*; Mitchell & Carman, 1926). Three litters of four young growing female rats were used in each experiment;

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each rat received each diet in turn according to a Latin-square design. Full experimental details will be found in our earlier publications (Henry, Kon & Watson, 1937; Henry, Kon, Lea & White, 1948). In Exp. 9 the periods for the collection of excreta were reduced from 6 to 4 days on the advice of Drs A. P. de Groot and C. Engel of Utrecht (private communication), who found the procedure satisfactory. It had the advantage of appreciably reducing the length of the experiment.

Vitamin-free casein (Glaxo Laboratories Ltd) was used in Exp. 4 and vitamin-low casein (Genatosan Ltd) in Exps. 7, 9 and 14. Heated soya grits were used in Exp. 13.

Exp. 12. The protein-efficiency ratio of 100% extraction wheat flour with and without supplements (see Table 1) was determined by the growth method of Osborne, Mendel & Ferry (1919). Six litters of eight newly weaned male rats were used; further details were as given by Henry *et al.* (1948).

Experiments with vitamin B_{12}-deficient rats

Litters of male rats were weaned on to one of the vitamin B_{12}-deficient diets (Table 2) and kept on it for a minimum depletion period of 4 weeks. Diet A was used in Exps. 1 and 2 only. It contained dried thyroid, which caused some deaths among the animals. In all other experiments diet B was used. We have found in this laboratory (unpublished results) that rats vary markedly in their growth rate on vitamin B_{12}-deficient diets. When available, therefore, more litter-mates than finally required were weaned on to diets A or B, to make possible selection of the more uniform animals for the metabolic tests. Animals given vitamin B_{12} received 0·2 μg/day, as we found (unpublished results) that with this dose deficient rats grow satisfactorily. Our normal Latin-square design, in which all rats receive each diet in turn, could not be used in these experiments with diets with and without vitamin B_{12}. The arrangement of rats in each experiment and the necessary slight variations in experimental details between experiments are given below.

Exp. 1. Six pairs of male rats from four litters were used. The casein ('grain curd') was prepared in the laboratory by the method of Clark, Zoller, Dahlberg & Weimar (1920) and was shown by chick tests to be free from the animal protein factor.

Exp. 2. Six litters of four male rats were used, one rat from each litter being given one of the four diets (Table 1). DL-Methionine (0·5%) was added to the casein, as previous work on milk (Henry & Kon, 1953) and the data of Block & Mitchell (1946–7) led us to consider that this addition should cover the sulphur amino-acid deficiency of casein. Glaxo vitamin-free casein was used.

Exp. 3. Three litters of four male rats were used, and there were two experimental periods. One animal from each litter received one of the diets (Table 1) during the first experimental period. During the second the animal that had been given the casein diet now received the casein diet supplemented with methionine, and vice versa; similarly, the animals given the vitamin B_{12} supplement were changed over. Glaxo vitamin-free casein was again used, but was first extracted three times with 70% ethanol to remove any traces of vitamin B_{12}. As in Exp. 2, the casein was supplemented with 0·5% DL-methionine.

Exps. 5 *and* 6. Six litters of three male rats were used in each experiment. In both,

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one rat from each litter was given the diet containing casein, casein + methionine or casein + homocysteine and choline. In Exp. 5 the last group received a further supplement of vitamin B_{12}. In these and subsequent experiments the methionine supplement to casein was increased to 1% as, contrary to expectation, the lower supplement did not significantly increase in Exps. 2 and 3 the biological value of the casein for the deficient rats (see Table 3). Genatosan low-vitamin casein was used.

Exp. 8. Six litters of four male rats and Genatosan low-vitamin casein were used. The experimental period was reduced to 4 days as in Exp. 9 (see p. 43).

Exp. 10. Six litters of three male rats were used for the tests with 100% extraction wheat flour.

Exp. 11. Five litters of seven male rats were used and the protein-efficiency ratio of 100% extraction wheat flour with or without various supplements (Table 2) was determined by the growth method of Osborne *et al.* (1919).

RESULTS

Effect of vitamin B_{12} on the biological value of casein

The results of these experiments (nos. 1–9) are given in Table 3. They show that with vitamin B_{12}-deficient rats the addition of the vitamin had no consistent effect on the biological value of casein. Thus, in Exp. 1 there was a significant increase, in Exp. 2 there was no effect and in Exp. 3 the increase was not statistically significant. Somewhat surprisingly the addition of 0·5% methionine to casein did not significantly increase its biological value for the deficient rats (Exps. 2 and 3), although the increase was significant for the normal rats (Exp. 4). In Exp. 3, but not in Exp. 2, the addition of the vitamin to casein supplemented with methionine significantly increased the biological value. For the normal rats (Exp. 4) the biological value of casein alone or with methionine was not affected by the addition of vitamin B_{12}. As already mentioned, in Exps. 5–7 the methionine addition to casein was increased to 1% and the biological value of casein was significantly increased, by some 12%, both with the normal and the deficient animals; a similar increase was observed for both groups when 1% homocysteine and 0·5% choline were added to casein with or without a supplement of vitamin B_{12}. In Exp. 8 (deficient rats) homocysteine had no effect on the biological value of casein unless vitamin B_{12} was given, when the increase did not differ significantly from that observed with the methionine supplement; for the normal rats a similar increase was obtained with homocysteine, whether alone or with vitamin B_{12} (Exp. 9); in this experiment the effect of the addition of 1% methionine was greater than in Exps. 5–8.

It will be noticed that the biological values obtained for the unsupplemented casein differed markedly from experiment to experiment. We periodically observe such differences between experiments, and consider that they are due to differences in response between different batches of rats. Relative values are, however, reproducible, and, for this reason we normally include a reference protein in all experiments. To facilitate comparisons between experiments, percentage differences from the biological value of casein caused by the various supplements are given in parentheses in Table 3.

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Table 3. *Effect on the biological value and true digestibility of casein of added methionine, or homocysteine and choline or homocysteine alone, each with or without a supplement of 0·2 μg vitamin B_{12} daily. Experiments with vitamin B_{12}-deficient and normal rats*

Addition to casein	Vitamin B_{12} supplement	Vitamin B_{12}-deficient rats: Exp. no.	No. of rats	Biological value	True digestibility	Normal rats: Exp. no.	No. of rats	Biological value	True digestibility
None	−	1	6	83·0 (100)	99·9				
None	+		6	90·0 (108)	99·3				
			S.E. (3 d.f.)	± 1·51	± 0·50				
None	−	2 and 3	6	84·3 (100)*	100·0*	4	12	79·4 (100)	97·8
				77·7 (100)†	98·8†				
None	+		6	83·6 (99)*	100·0*		12	78·4 (99)	97·3
				82·4 (106)†	97·3†				
0·5 % DL-methionine	−		6	89·1 (106)*	99·9*		12	85·5 (108)	98·3
				81·5 (105)†	98·0†				
0·5 % DL-methionine	+		6	89·0 (106)*	100·0*		12	86·8 (109)	98·2
				89·5 (115)†	97·3†				
			S.E. (15 d.f.)	± 1·75*	± 0·04*		S.E. (18 d.f.)	± 1·17	± 0·43
				± 1·81†	± 0·48†				
None	−	5 and 6	6	84·9 (100)‡	98·7‡	7	12	76·7 (100)	98·8
				85·4 (100)§	99·1§				
1 % DL-methionine	−		6	95·1 (112)‡	99·7‡		12	85·5 (112)	99·6
				95·5 (112)§	99·5§				
1 % homocysteine, 0·5 % choline	−		6	—	—		12	85·5 (112)	99·3
				94·0 (110)§	99·7§				
1 % homocysteine, 0·5 % choline	+		6	94·3 (111)‡	99·9‡		12	84·3 (110)	99·3
			S.E. (10 d.f.)	± 1·10‡	± 0·39‡		S.E. (18 d.f.)	± 1·22	± 0·29
				± 1·79§	± 0·27§				
None	−	8	6	68·5 (100)	98·7	9	12	74·0 (100)	99·3
1 % DL-methionine	−		6	78·0 (114)	99·6		12	86·6 (117)	99·7
1 % homocysteine	−		6	70·2 (102)	99·9		12	81·8 (111)	99·7
1 % homocysteine	+		6	76·3 (111)	98·8		12	81·4 (110)	99·2
			S.E. (15 d.f.)	± 1·88	± 0·49		S.E. (18 d.f.)	± 1·09	± 0·34

Figures in parentheses are values referred to casein = 100.
* Exp. 2.
† Exp. 3.
‡ Exp. 5.
§ Exp. 6.

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Table 4. *Effect on the biological value, true digestibility and protein-efficiency ratio of 100% extraction wheat flour or of soya-bean meal of added amino-acids, with and without a supplement of 0·2 μg vitamin B_{12} daily. Experiments with vitamin B_{12}-deficient and normal rats*

Addition to protein source	Vitamin B_{12} supplement	Vitamin B_{12}-deficient rats: Exp. no.	No. of rats		Biological value	True digestibility	Protein-efficiency ratio	Normal rats: Exp. no.	No. of rats	Biological value	True digestibility	Protein-efficiency ratio
			100% extraction wheat flour									
None	−	10 and 11	6*	5†	63·9	91·5	1·64	12	6	—	—	1·43
	+		6*	5†	62·7	91·1	1·90		6	—	—	1·51
	−		6*	5†	64·4	91·0	1·72		6	—	—	1·56
DL-methionine	+		S.E. (10 d.f.) ± 1·54			± 1·03	—		6	—	—	1·54
DL-methionine	−			5†	—	—	2·07		6	—	—	2·01
1% L-lysine	+			5†	—	—	2·44		6	—	—	2·12
1% L-lysine	−			5†	—	—	2·13		6	—	—	2·20
1% L-lysine, 0·5% DL-methionine	+			5†			2·22		6	—	—	2·33
1% L-lysine, 0·5% DL-methionine						S.E. (22 d.f.) ± 0·07					S.E. (35 d.f.) ± 0·06	
			Soya-bean meal									
None	−		—	—	—	—	—	13	12	72·3	85·0	—
None	+		—	—	—	—	—		12	72·2	84·6	—
1·6% DL-methionine	−		—	—	—	—	—		12	81·7	86·3	—
1·6% DL-methionine	+		—	—	—	—	—		12	81·7	85·2	—
									S.E. (18 d.f.) ± 1·92		± 1·20	

* Exp. 10. † Exp. 11.

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Effect of vitamin B_{12} on the utilization of vegetable proteins

The results of these experiments are given in Table 4. They show that for the vitamin B_{12}-deficient rats (Exps. 10 and 11) the biological value of 100% extraction wheat flour was not affected by supplements of methionine or of vitamin B_{12}. The protein-efficiency ratio of the flour, alone or supplemented with lysine, was, however, significantly increased when vitamin B_{12} was given. With normal rats (Exp. 12) vitamin B_{12} had no effect on the protein-efficiency ratio of the flour, either alone or supplemented with methionine or lysine or with both, but the addition of methionine caused a significant increase with the flour supplemented with lysine.

With normal rats (Exp. 13), the addition of vitamin B_{12} had no effect on the biological value of soya alone or supplemented with methionine. Methionine, as would be expected, caused a significant increase.

Effect of vitamin B_{12} on the biological value of casein at 8% and at 16% levels of protein intake

The results of this experiment (Exp. 14) are shown in Table 5. Vitamin B_{12} had no effect on the biological value of casein at either level of intake.

Table 5. *Biological value and true digestibility of casein at 8% and 16% levels of protein intake with or without a supplement of 0·2 μg vitamin B_{12} daily. Experiment with normal rats*

Exp. no.	No. of rats	Level of casein	Vitamin B_{12} supplement	Biological value	True digestibility
14	12	8% protein	−	79·8	99·1
	12	8% protein	+	82·2	99·2
	12	16% protein	−	65·6	98·8
	12	16% protein	+	64·4	98·3
			S.E. (18 d.f.)	±1·22	±0·33

Vitamin B_{12} and true digestibility of protein

In none of the metabolic experiments (1–10, 13 and 14) was the true digestibility altered by the addition of vitamin B_{12}.

DISCUSSION

Our most significant finding was that homocysteine increased the biological value of casein in the presence of vitamin B_{12}, but not in its absence (Exps. 8 and 9). With the vitamin 1% homocysteine was as effective as 1% methionine, showing that, in the presence of a suitable acceptor, vitamin B_{12} is involved in the synthesis of methyl groups. Our quantitative study of protein metabolism in rats thus provides further support to the earlier evidence of this specific function of vitamin B_{12}, which has been demonstrated in growth experiments (Stekol & Weiss, 1950; Jukes, Stokstad & Broquist, 1950; Young, Norris & Heuser, 1954; Chang & Johnson, 1955; Johnson, Firth & Mistry, 1955), with radioactive tracers (Stekol, Weiss & Weiss, 1952;

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Arnstein & Neuberger, 1953, Arnstein, 1955) and microbiologically (Johnson, Holdsworth, Ford, Porter & Kon, 1955).

It is now generally agreed (Arnstein & Neuberger, 1953; Firth, Mistry, James & Johnson, 1954; Mistry, Vadopalaite, Chang, Firth & Johnson, 1955; Stekol, Weiss, Smith & Weiss, 1953; Young *et al.* 1954) that vitamin B_{12} plays no part in transmethylation. The results of Exps. 5–7 support this view. They show that addition of 1% homocysteine and 0·5% choline increased the biological value of casein for the deficient rat to the same extent as 1% methionine. Hence vitamin B_{12} is not necessary for the transfer of methyl groups from choline to homocysteine.

In Exp. 1, already briefly reported (Henry & Kon, 1951), the biological value of casein was significantly improved by a supplement of vitamin B_{12}, but we could not confirm this finding in two experiments with deficient rats (Exps. 2 and 3). We must conclude, therefore, in agreement with our other findings and with present views, that vitamin B_{12} is not involved in protein utilization as such.

It has been mentioned (p. 44) that in Exps. 2 and 3 the increase in the biological value of casein caused by the addition of 0·5% methionine was significant for normal rats (Exp. 4), but not for the deficient rats. It is not possible to say whether this finding indicates a slightly higher methionine requirement by the vitamin B_{12}-deficient rat or whether it is due to differences in response between batches of rats (cf. p. 44). The latter is perhaps the more likely explanation, as with normal rats a greater increase in the biological value of casein resulted from addition of 1% methionine in Exp. 9 than in Exp. 7.

The remaining experiments described in this paper further demonstrate that vitamin B_{12} does not specifically improve the utilization of proteins (cf. Henry & Kon, 1951). Exps. 10–12 (Table 4) were done to test its effect on the utilization of a protein deficient in lysine, namely that of 100% extraction wheat flour. In these experiments the protein-efficiency ratio of flour, alone or supplemented with lysine, was significantly improved by vitamin B_{12} (Exp. 11), but this apparent increase was not confirmed in a balance experiment done at the same time (Exp. 10). As the protein-efficiency ratio expresses the gain in weight per unit protein ingested, its significance hinges on the composition of the weight gain. Several authors (Black & Bratzler, 1952; Knoebel & Black, 1952; Ling & Chow, 1952) have shown that deficient rats given vitamin B_{12} gain weight largely by deposition of fat, probably because of increased synthesis of choline (cf. Arnstein, 1955). It is therefore probable that the effect of vitamin B_{12} on the protein-efficiency ratio in Exp. 11 was spurious and should be discounted. In the experiment with normal rats (Exp. 12), when vitamin B_{12} would not be expected to stimulate growth, the protein-efficiency ratio of flour, whether alone or supplemented with amino-acids, was not improved by its further addition. In this experiment methionine significantly increased the protein-efficiency ratio of the flour supplemented with lysine, but not that of the unsupplemented flour. This finding indicates that, as one would expect, methionine, which with valine is the secondary limiting amino-acid in flour, only becomes an effective supplement when the primary lysine deficiency has been at least partly overcome.

We can sum up the results of these fourteen experiments as providing further

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evidence that vitamin B_{12} is involved in the synthesis of methyl groups, but that it does not play a part in transmethylation. There is no proof that it is in any other way involved in protein metabolism.

SUMMARY

1. The effect of vitamin B_{12} on protein metabolism has been studied in a series of experiments with vitamin B_{12}-deficient and normal rats.

2. For deficient rats the biological value of casein was increased to the same extent by the addition of 1% methionine as by the addition of 1% homocysteine and a vitamin B_{12} supplement. Homocysteine was ineffective in the absence of the vitamin. It is concluded that vitamin B_{12} is involved in the synthesis of methyl groups.

3. With deficient rats the addition of 1% homocysteine, together with 0·5% choline, in the presence or absence of vitamin B_{12}, increased the biological value of casein to the same extent as 1% methionine. This finding demonstrates that the vitamin plays no part in transmethylation.

4. In two out of three experiments the addition of vitamin B_{12} had no effect on the biological value of casein determined on deficient rats.

5. For deficient rats vitamin B_{12} did not increase the biological value of the protein in wheat flour of 100% extraction. The protein-efficiency ratio of the flour alone, or supplemented with lysine or with both lysine and methionine, was increased by the addition of vitamin B_{12}; it is considered that this finding may be spurious, as the method hinges on the composition of the weight gain in relation to protein intake.

6. With normal rats the addition of vitamin B_{12} had no effect on the protein-efficiency ratio of 100% extraction flour alone or supplemented with lysine or methionine or both.

7. With normal rats vitamin B_{12} did not increase the biological value of soya or of casein at a high (16%) level of protein intake.

8. It is concluded that vitamin B_{12} plays a part in the synthesis of methyl groups, but that it neither functions in transmethylation nor is involved in protein utilization as such.

We are grateful to Dr M. E. Coates for preparing the casein used in Exp. 1. We wish to thank Miss V. Glover and Miss M. R. Cooling for help with the nitrogen analyses and the feeding of the rats.

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Perspectives in Nutrition

It is hoped that Perspectives in Nutrition will review the literature selectively, interpret it moderately and present a spectrum of ideas that will serve as a continual stimulation to nutritional research applied to medical problems.

Nutritional Requirements for Vitamin B_{12} and Folic Acid[1,2,3]

V. HERBERT

THE FOOD AND NUTRITION BOARD of the National Academy of Sciences–National Research Council has, since 1940, developed formulations of daily nutrient intakes judged adequate for the maintenance of good nutrition in the population of the United States. As they state (1) "These formulations were designated 'Recommended Dietary Allowances' in order to indicate that they were value-judgments based on the existing knowledge of nutritional science and subject to revision as new knowledge became available. The allowances are intended to serve as goals toward which to aim in planning food supplies and as guides for the interpretation of food consumption records of groups of people. Actual nutritional status of groups of people or individuals must be judged on the basis of physical, biochemical, and clinical observations combined with observations on food or nutrient intakes. If the recommended allowances are used as reference standards for interpreting records of food consumption, it should not be assumed that food practices are necessarily poor or that malnutrition exists because the recommendations are not completely met." As stated in the appendix of that book, "The purpose for establishing a national dietary standard is not the same in all countries. Therefore, some variation in nutrient allowances from country to country is to be expected. At the same time, it must be recognized that the "reference" individual will vary from country to country. Furthermore, even in instances where there are presumed to be similar objectives among countries as to the purpose and usefulness of proposed standards, it can be seen that there is by no means uniform agreement as to the nutrient allowances considered desirable as national guides. Standards are also subject to revision as newer knowledge becomes available." That same appendix lists the "reference male" as having a weight ranging from 53 kg in the Philippines to 73 kg in South Africa, and the "reference

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female" as having a weight ranging from 15 kg in India and the Philippines to 60 kg in South Africa.

In light of the above, and to achieve some basis for an internationally acceptable standard, before discussing nutritional requirements for vitamin B_{12} and folic acid, it is necessary to state certain arbitrary definitions: nutritional requirement will be considered as identical to minimal daily adult requirement; minimal daily adult requirement will be considered as that quantity of nutrient which will sustain normality in healthy adult male subjects without excess deposits of the nutrient (i.e., subjects with "low" body stores); normality with respect to a given nutrient will be considered as absence of any clinical symptoms or signs and absence of any laboratory evidence of deficiency of the nutrient. Implicit in use of the word normal is the understanding that it is a relative term with no absolute meaning (2). The dictionary definition of the word normal is that it is the usual condition, degree, quantity, or the like; average; mean. The average height and weight of the Asian adult male are considerably less than those of the North American adult male, which are considerably greater than those of the adult North American female (1). The height and weight of infants are considerably less than those of adults; however, the lesser nutritional requirement of infants due to their lesser total body mass is partly offset by their larger nutritional requirement per unit body mass because of growth. Physiological requirements for any nutrient vary from day to day in proportion to variations in total body metabolic rate. Metabolic rate is varied by many internal factors such as stress, infection, and pregnancy, as well as by many external factors such as temperature, humidity, altitude, and diet patterns (1, 3). Finally, the requirement for a given nutrient is defined in terms of the quantity of the nutrient actually absorbed and utilized; this may be considerably below the quantity of the nutrient that must be ingested, since only a variable portion of ingested nutrient is absorbed. In this connection, it is crucial to recognize that any structural or functional abnormality in the gastrointestinal tract may reduce nutrient absorption.

NUTRITIONAL REQUIREMENT FOR VITAMIN B_{12}

The minimal daily adult male requirement for vitamin B_{12} is about 0.1 μg. This is the quantity of vitamin B_{12} that, when administered parenterally daily as cyanocobalamin or coenzyme B_{12} to patients with vitamin B_{12} deficiency uncomplicated by other systemic diseases, will produce a gradual return toward hematologic normality, manifested by reticulocytosis and the gradual rise of hematocrit toward normal. It has not yet been determined whether that daily dose will produce complete return to hematologic normality, since the maximum length of such study was for 2 months of 0.1 μg of cyanocobalamin daily. Further, it has not yet been determined whether that dose of vitamin B_{12} daily will produce return to neurologic normality.

Although reticulocytosis occurs and hematocrit rises, serum vitamin B_{12} levels of vitamin B_{12}-deficient subjects are not always clearly raised by intramuscular injection of 0.1 μg of the vitamin daily for periods up to a month (4), but preliminary studies suggest that a gradual rise does occur when the parenteral dose is 0.2 μg daily. Daily parenteral doses of 1 μg of vitamin B_{12} raise the serum vitamin B_{12} level of such subjects into the clearly normal range within 2 weeks (see Fig. 64-3 of reference 5). Thus, the minimal daily adult requirement for vitamin B_{12} is definitely below 1 μg, is not below 0.1 μg, and will probably prove to be approximately 0.1 μg (and not above 0.2 μg). If 0.1 μg were much below the minimal daily require-

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ment, it might have sustained the hematocrit of vitamin B_{12}-deficient subjects, since the requirement to sustain a deficient state is less than the requirement to sustain a normal state, but it would probably not have raised it. The fact that 0.1 μg of vitamin B_{12} gradually raised the hematocrit of deficient subjects suggests that dose is close to, and perhaps equal to, the requirement to sustain normality.

A number of workers have estimated human requirements for vitamin B_{12} on the basis of whole-body turnover per day of tracer doses of vitamin B_{12} (6–9). Such studies put the minimal daily adult requirement in the range of 0.5–2.5 μg. This may be higher than the true requirement largely because most of these studies used normal subjects, i.e., individuals without vitamin B_{12}-deficiency disease (10). It is clearly true for vitamin B_{12} (9), and probably generally true for most (if not all) nutrients, that whole-body turnover per day is progressively less in quantity as the quantity of nutrient decreases. Heyssel et al. (9) suggest an obligatory rate of daily loss of vitamin B_{12}, which is 0.1% of the total body pool regardless of how large or small the pool may be. In a general sense, studies of the whole-body turnover rate per day of tracer doses of radioactive vitamin measure the quantity of vitamin required to maintain the status quo, whatever it may be, and do not measure the needs of the body to sustain normality.

The statement that the minimal daily adult requirement for vitamin B_{12} is approximately 0.1 μg does not mean such a dose is optimal therapy for vitamin B_{12} deficiency. This dose produces only very slow return toward normality. Rapid return to normality is produced by 1 μg of the vitamin daily; such a dose is recommended for therapeutic trials (5).

The optimal therapeutic dose of vitamin B_{12} is probably not much less than 1 μg daily and not much more than 40 μg daily. Urinary excretion increases logarithmically as the daily dose of vitamin B_{12} increases above 10 μg; much of such increases is lost in the urine (5–8). The optimal daily adult requirement for vitamin B_{12}, as for any nutrient, is unknown. As Goodhart (3) and others have noted, "it is practically impossible to devise a general food plan that will be just right for everyone. . . It is not at all certain, for example, that the nutritional requirements for maximum size, early maturity, active sex life and maximum muscular development are identical with those for maximum longevity."

Nutritional requirement for vitamin B_{12} may not necessarily be met by ingestion each day of food containing the bare minimum daily adult requirement of vitamin B_{12} as determined from food tables, such as those of the U.S. Department of Agriculture (11) although a diet containing (after cooking and other processing prior to ingestion) 1 μg of vitamin B_{12} daily is almost certainly adequate to sustain normality in healthy adult male subjects. One cannot assume total availability for absorption of vitamin B_{12} in food, although at least half of quantities up to 3 μg are probably absorbed (9). It is probable that nearly all food vitamin B_{12} is peptide bound and is only available for absorption after release from peptide bonds by gastric acid and/or intestinal alkali and/or alimentary tract enzymes (5). There are two mechanisms for vitamin B_{12} absorption (12, 13). One of these, the active, intrinsic factor-dependent mechanism, is physiologically predominant. It accounts for most of the 0.5–10 μg of vitamin B_{12} absorbed daily from the 1–100 μg of vitamin B_{12} found in most diets (11). It must be remembered that, while practically no vitamin B_{12} is destroyed by boiling in water at acid pH 4–6, about 90% is destroyed by such boiling at pH 8, unless protected by certain reducing substances (15). The intrinsic factor mechanism for absorption of vitamin B_{12} can efficiently transport into the blood stream a maximum of 2–3 μg of vita-

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min B_{12} in any one meal, but will transport this much from as little as 2–3 μg of food vitamin B_{12} (9, 12). This mechanism requires essentially normal gastric parietal cells, which secrete intrinsic factor (16), and an essentially normal ileum (since the ileum is the major site of intrinsic factor-mediated vitamin B_{12} absorption) (17).

The other mechanism for vitamin B_{12} absorption is passive (probably diffusion), is operative primarily in the presence of large quantities of vitamin B_{12} such as may be found in a meal of almost raw liver, allows absorption of only about 3% of the vitamin presented to the intestine, and probably occurs along the entire length of the small bowel (18). The figure of 3% absorption is based on the assumption (19) that the amount found in the urine on Schilling-type testing (1%) (12) represents about one-third of the total absorbed.

Cessation of absorption of vitamin B_{12} due to intrinsic factor lack or small bowel disease leads to much more rapid overt vitamin B_{12} deficiency than does dietary deprivation of the vitamin. The person whose diet lacks the vitamin almost completely reabsorbs the 0.5–5 μg (5–8) of vitamin B_{12} he secretes daily in his bile, whereas the subject who lacks intrinsic factor or has ileal dysfunction does not. Failure to reabsorb vitamin B_{12} secreted in the bile results in clinical vitamin B_{12} deficiency within 3–6 years. The subject with dietary lack of vitamin B_{12} efficiently reabsorbs practically all of the vitamin secreted in his bile and thus only gradually develops overt vitamin B_{12} deficiency, over a period of 10–20 or more years. This, plus absorption of the small amounts of vitamin B_{12} present in the root nodule microorganisms of legumes (20), explains why strict vegetarians who eat no animal protein develop vitamin B_{12}-deficiency disease only slowly, after decades of dietary deprivation. They have an almost perfect enterohepatic circulation of vitamin B_{12}, and since there is no evidence that vitamin B_{12} is degraded in the human body they only slowly lose their body stores (5).

NUTRITIONAL REQUIREMENT FOR FOLIC ACID

The minimal daily adult requirement for folic acid is about 50 μg (5, 21, 22). This is the quantity of folic acid that, when administered orally or parenterally daily as pteroylglutamic acid to patients with folate deficiency uncomplicated by other systemic diseases, will produce a relatively rapid return toward hematologic normality, accompanied by reticulocytosis and rise of hematocrit toward normal. Serum folate levels do not rise appreciably on this dose of folic acid given for periods up to a month, despite conversion of bone marrow morphology from megaloblastic to normoblastic and marked hematologic improvement (23). However, serum folate levels do rise to normal within a month when the daily dose of folic acid is 0.1 to 0.2 mg (*patient FB* in reference 21).

Supporting 50 μg of folic acid as in the range of the minimal daily adult requirement are several other studies: *1*) Three healthy adult female medical research technicians were given a diet containing approximately 5 μg of folate daily, supplemented with a single daily oral tablet of pteroylglutamic acid. In the space of slightly more than a month, a clear fall of the normal *Lactobacillus casei* serum folate level of 10.3 ng/ml to a subnormal level of 4.2 ng/ml occurred in the technician whose daily tablet contained 25 μg of pteroylglutamic acid; however, the normal serum folate level of 6.5 ng/ml of the subject receiving a daily tablet containing 50 μg of pteroylglutamic acid daily was essentially the same (6.2 ng/ml) at the end of the study, and the normal serum folate level of 11.0 ng/ml in the subject receiving a daily tablet containing 100 μg of pteroylglutamic acid was also essentially the same (9.4 ng/ml) at the end of the study (21). *2*) Fleming et al. (25) noted that a supplement of ap-

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proximately 50 μg/day of food folate prevented the appearance of elevated formiminoglutamate in the urine of a patient on a folate-deficient diet that had led to abnormally high formiminoglutamate excretion in the urine of three patients who did not receive the supplement. *3*) A healthy adult male with normal tissue folate stores in the range of 7.5 mg (26) developed megaloblastic anemia after 137 days of a diet containing only about 5 μg of folate daily (27). Since erythrocyte folate had fallen from the normal value of 160 ng/ml at the start of the study to 16 ng/ml at the time megaloblastosis appeared, one can roughly assume loss of 90% of tissue stores. If one divides 90% of 7.5 mg into 137 days, one gets a daily utilization of approximately 50 μg, plus about 5 μg in the daily diet. *4*) A woman with folate-deficiency megaloblastic anemia was sustained on a diet almost devoid of folate for 2 years, supplemented by 75 μg of pteroylglutamic acid daily. On this diet, macrocytosis disappeared after 214 days and neutrophil nucleus hypersegmentation disappeared after 282 days. This dose was probably greater than required to sustain normality, since there was evidence that her tissue folate stores were gradually replenished on this dose (28).

Setting the minimal daily adult folate requirement as in the range of 50 μg does not preclude the possibility that this requirement may be hinged to body weight, as well as to body metabolic rate, and therefore may vary with variations in the normal range of these two measurements. For example, Fig. 1 shows evidence for a reticulocyte response in a patient who weighed 38 kg, when given less than 50 μg of folate daily. Folate requirement may also vary with the nature of the folate. Figure 2 suggests a greater response to 50 μg of folinic acid after prior treatment with 50 μg of folic acid (100 μg of DL-folinic acid is equal to approximately 50 μg of the metabolically active form of folinic acid).

In the studies of Sheehy et al. (29, 30) and of Druskin et al. (31) hematologic response was obtained to 25 μg of pteroylglutamic acid daily, unless serum vitamin B_{12} levels were below 100 pg/ml. However, in those studies, the pteroylglutamic acid was superimposed on normal diets. It is a reasonable assumption that, despite the presence of sprue in the subjects of Sheehy et al., approximately another 25 μg of folate were absorbed from those diets. From their studies, Sheehy et al. felt that "man's minimum daily requirements for folic acid may be less than 100 μg" (29). In the studies with 50-μg therapeutic trial doses (21–23, 27), diets were almost devoid of folate (26) and thus it was possible to delineate daily requirement more sharply.

The recommended dose of pteroylglutamic acid for therapeutic trials in subjects with suspected folate deficiency who do not have an increased folate requirement is 100 μg daily orally or intramuscularly (24). This dose produced a gradual rise toward normal in the serum folate level over a period of 2–4 weeks, accompanied by a sharp return toward hematologic normality.

The minimal daily adult requirement for folic acid is increased by any increase in the daily metabolic rate or cell turnover rate (5), as in hyperthyroidism (32), pregnancy (33), and hemolytic anemia (34). This increased requirement is probably in the range from 100 to 300 μg daily, as suggested by the studies of Lindenbaum and Klipstein (34), Willoughby (35), and Alperin et al. (33). Hopefully it will not be greater than 300 μg, because daily doses of 100 μg may produce hematologic response in patients with vitamin B_{12} deficiency and no evidence of folate deficiency (24) and thus lead to the erroneous diagnostic conclusion that vitamin B_{12} deficiency is not present. Therapeutic trials with 0.1 and 0.2 mg of pteroylglutamic acid daily did not produce hematologic response in two women with folate-deficiency megaloblastic

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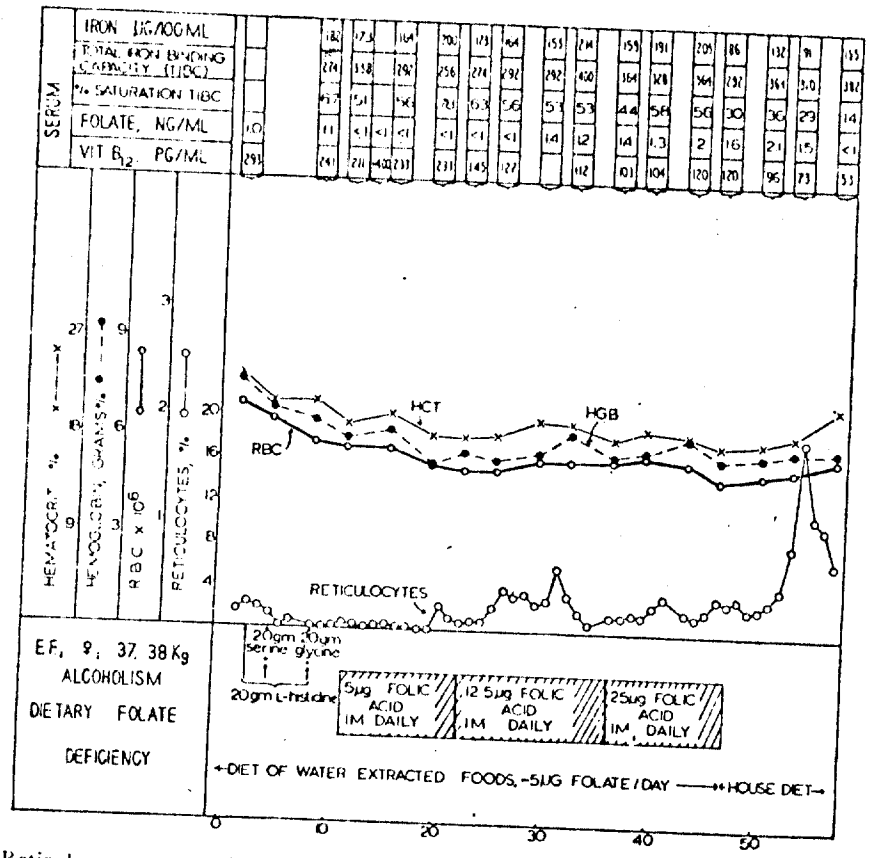

FIG. 1. Reticulocyte response (without rise in hematocrit) to less than 50 µg of pteroylglutamic acid intramuscularly daily, by a patient who weighed 38 kg, while ingesting a diet containing approximately 5 µg of total folate per day. Patient studied with L. W. Sullivan in February and March of 1962.

anemia of pregnancy (33), and produced poor responses in two women postpregnancy (Metz, personal communication), with a secondary reticulocyte response when the dose was raised to 0.4 mg daily. Pritchard (36) had previously observed a poor response to 0.4 mg of pteroylglutamic acid daily but a good response to 1 mg of folic acid daily in a pregnant subject. Figure 3, from a study with L. W. Sullivan, demonstrates the failure of a woman in the third trimester of pregnancy to respond to a single dose of 100 µg of folic acid, although she subsequently had a maximal response to 1 mg/day of folic acid. This response was accompanied by a sharp rise of the serum folate level to the upper limit of normal. The average Asian pregnant woman, being smaller than the average North American pregnant woman, may respond more frequently to therapeutic trial with 0.1 mg daily (37).

The nutritional requirement for folic acid can probably not be met by ingestion each day of food containing the bare minimum daily adult requirement as determined from food tables. This is because most, if not all, food folate is in conjugated form and probably requires deconjugation before absorption (5). Studies of the absorbability of folate in foods are needed; these can be carried out along lines such as have been indicated elsewhere (38). Baumslag and Metz (39) have demon-

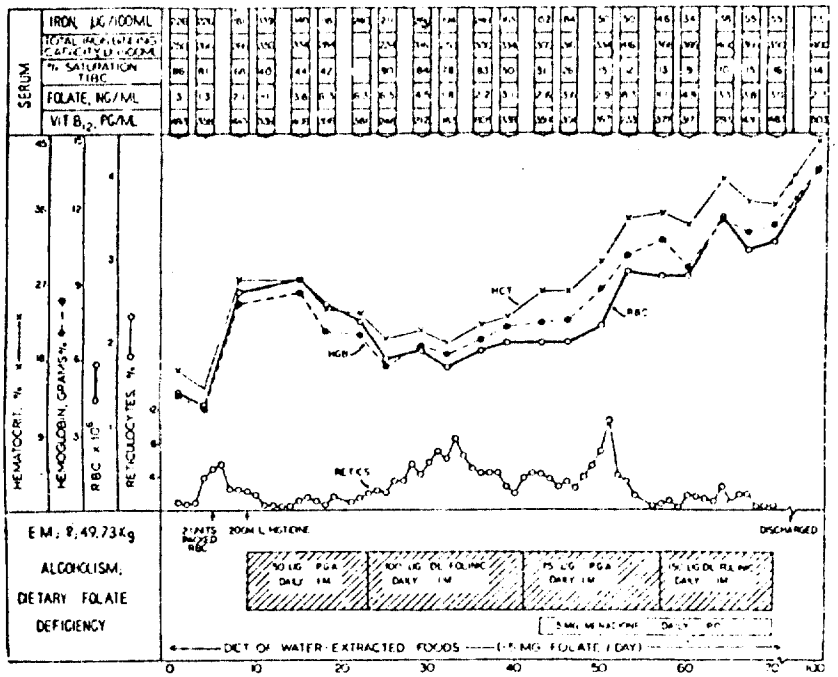

FIG. 2. Seemingly greater hematologic response to 50 µg of folinic than to the same quantity of folic acid, in a patient on a diet containing approximately 5 µg of folate per day. The 100 µg of DL-folinic acid is equal to approximately 50 µg of metabolically active folinic acid. Patient studied with L. W. Sullivan in October and November of 1963.

strated hematologic response of a patient, with megaloblastic anemia in the puerperium, to eating three whole lettuces daily for 6 days.

Purified crystalline folate monoglutamates appear to be absorbed rapidly and almost completely, primarily from the proximal small intestine, although they are capable of being absorbed from the entire length of the small intestine (40–42). In rats, and presumably in man, this absorption is an active process for small ("physiologic") quantities of the vitamin, but it is probably by diffusion for large ("supraphysiologic") quantities (43, 44). This probable dual mechanism for folate absorption is somewhat analogous to that for vitamin B_{12} absorption. The percentage of the total folate in liver, yeast, and egg yolk that is absorbed is very high; but the absorbability of the forms of folate in most other foods is probably in the range of about 10%. Because of the limited absorption of food folate, only about 5–10 µg of free folate activity for *L. casei* appears in the daily urine of normal individuals ingesting an average diet (5). When there is structural or functional damage in the upper small bowel, such as in tropical sprue, much of the food folate is so poorly absorbed that up to 1,500 µg of food folate given by mouth will often not produce a hematologic response. On the other hand, absorption of crystalline folic acid is so complete, even in the presence of the otherwise generalized malabsorption of tropical sprue, that some such patients may have hematologic responses to as little as 25 µg of crystalline folic acid after failure to respond to food containing up to 1,500 µg of folate (29, 30).

From 50 to 95% or more of food folate

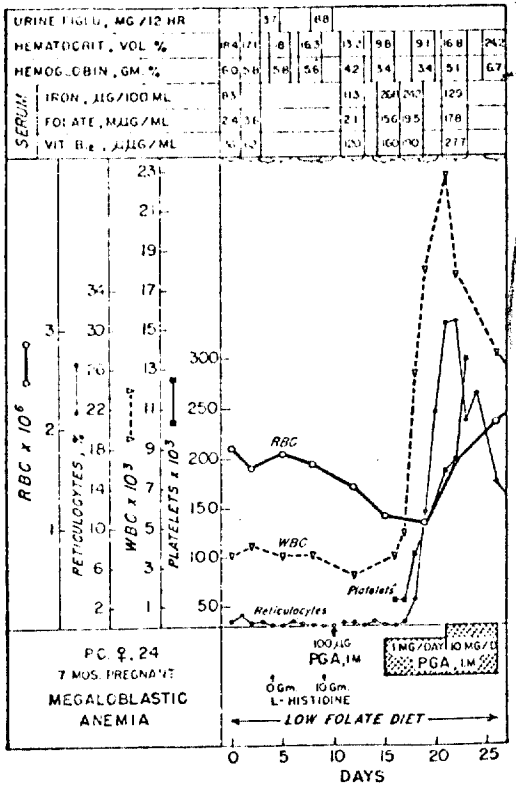

FIG. 3. Dramatic hematologic response to 1 mg of folic acid intramuscularly daily after failure to respond to 0.1 mg as a single dose, in a patient in the 7th month of her fourth pregnancy in 6 years. Note also the rise in serum vitamin B_{12} level on therapy with folate. Patient studied with L. W. Sullivan in January and February of 1962.

may be destroyed in cooking, canning, or other forms of processing (5, 26). Significant protection against oxidative destruction is afforded by the presence of ascorbate (26); paradoxically, ascorbate causes gradual destruction of aqueous solutions of vitamin B_{12} (15). Because so large a percentage of food folate may be lost by processing, the measure of daily dietary folate ingestion is only valid when made after all processing is completed and the food is about to be ingested.

It should be noted that the minimal daily infant folate requirement may be almost as much as that of the adult, presumably because of the sharply increased needs for growth (15, 46).

SUMMARY

The minimum daily adult vitamin B_{12} requirement is in the range of 0.1 µg, and the minimal daily adult folate requirement is in the range of 50 µg. Vitamin B_{12} is not catabolized, but folate is (47); it is partly for this reason that the requirement for folate is much greater than for vitamin B_{12}. Folate requirement is sharply increased (perhaps up to sixfold) by conditions producing increase in metabolism of one-carbon units, such as hyperthyroidism, pregnancy and hemolytic anemia; increases in vitamin B_{12} requirement brought about by increased metabolic rate are considerably less striking. Measurements of serum (48) and erythrocyte (49) folate activity will continue to be major guides in assessing folate requirements.

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Vitamin B_{12} Turnover in Man

The Assimulation of Vitamin B_{12} from Natural Foodstuff by Man and Estimates of Minimal Daily Dietary Requirements

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IN previous reports[1-3] we have described the rate of loss from man of Co^{60}-labeled vitamin B_{12}. These investigations, made on both healthy subjects and patients with pernicious anemia, indicate that the rate of loss of body vitamin B_{12} is approximately 0.1 per cent of body stores per day. Most importantly the rate of loss, but not the absolute quantity lost, appears to be independent of the size of the body stores. We have termed this apparently fixed rate of loss an "obligatory loss."

One of the problems inherent in applying such a model is the relatively high estimate of absolute daily loss of vitamin which results when normal subjects are considered. As an example, assuming the total body content of vitamin B_{12} to be 2,000 to 5,000 μg. in an adult man,[4,5] then 2 to 5 μg. of vitamin would be lost daily. If a reasonably steady state exists in the adult, with regard to the body vitamin B_{12} stores, 2 to 5 μg. of vitamin B_{12} need to be replaced daily from dietary sources. The studies to be described demonstrate that daily assimilation of 2 to 5 μg. of vitamin B_{12} is probable in normal adults eating the usual American diet and that estimated daily gains of vitamin B_{12} correlate well with our previous estimates of loss. The results along with other clinical observations, which will be cited, afford an opportunity to estimate the daily dietary requirement of vitamin B_{12}.

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MATERIALS AND METHODS

*Preparation of Food Containing Co^{60}-Labeled Vitamin B_{12}**

The vitamin B_{12} content of ruminants is derived from the vitamin synthesized by the bacterial flora in the rumen and subsequently absorbed further down the gastrointestinal tract of the animal.[6] For this reason sheep were chosen as the source of meat containing Co^{60}-labeled vitamin B_{12}. Two ewes were fed a purified diet as described by Wright and Bell[7] except that cobalt was omitted. After two weeks on this diet, they received 1 mc. per day of $Co^{60}Cl_2$ (specific activity 45 mc. per mg.) orally for five consecutive days. Three weeks after cessation of feeding of the radioactive material, urinary excretion of Co^{60} was negligible and the animals were slaughtered in a conventional manner. The lean meat was stripped from the bones, ground and mixed well and made into weighed 100 gm. meat patties. The liver and other organs were removed and wrapped for separate storage. All the material was stored at −20°c. Unfortunately, the bulk of the lean meat was lost due to failure of a freezer. The meat patties used in this study and all the liver had been placed in other freezer units. As a result of the loss, the data are less in number than originally planned.

* Labeled meat was prepared at the University of Tennessee Agricultural Research Laboratory, Oak Ridge, Tennessee.

TABLE I

Total Co^{60} Radioactivity of Liver and Muscle Meat and Per Cent Present as Ionic Cobalt

Meat	Total Radioactivity (μc./100 gm.)	Ionic Co^{60} (%)*
Liver	7.7	3.6
	6.3	2.5
Muscle	0.035	6.4
	0.025	7.9

* Per cent of total radioactivity present.

Analysis of Meat for Ionic Cobalt, Vitamin B_{12} and Co^{60} Activity

Prior to use, the meat and liver were assayed for radioactivity, ionic cobalt and vitamin B_{12} content, and for the identity of radioactivity with cyanocobalamin after extraction-treatment with cyanide. Ionic cobalt was assayed utilizing a reverse isotope dilution technic described by Rosenblum and colleagues.[8] The vitamin B_{12} content was determined microbiologically utilizing Euglena gracilis var. bacillus[9] and selected assays were made utilizing Lactobacillus leichmanii.[10] The association of label with compound convertible to cyanocobalamin was determined by extraction and reverse isotope dilution analysis according to the method of Bacher, Boley and Shonk.[11] Table I summarizes the analysis of radioactivity expressed in microcuries per 100 gram of meat and liver. Ionic cobalt content was as low as 2.5 per cent in liver, with the highest value being 7.9 per cent in lean meat. The total radioactivity of liver and lean meat per gram varied widely. Paralleling this were wide differences in total vitamin B_{12} activity between liver and lean meat (Table II). As a result the ratios of radioactivity in microcuries to micrograms of vitamin B_{12} (specific activity) are quite similar and suggest near equilibrium of labeled and nonlabeled vitamin B_{12} at the end of three weeks.

TABLE II

Content and Specific Activity of Co^{60}-Vitamin B_{12} in Liver and Muscle Meat

Meat	Vitamin B_{12} Content (μg./gm.)	Specific Activity* (μc./μg.)
Liver	1.48	0.05
Meat	1.12†	0.03

* Microcuries of Co^{60} per microgram of vitamin B_{12}.
† Micrograms per 100 grams.

An aliquot of liver was treated with sodium nitrite and potassium cyanide, extracted and the distribution coefficient of the purified cobalamins determined in a system of benzyl alcohol and water.[12] It is evident (Table III) that the distribution coefficient determined by radioactivity or by color were essentially identical and corresponded to that for cyanocobalamin (1.21). This was further checked by chromatography of the purified extract with known standards, compound A, compound B, pseudovitamin B_{12}, hydroxycobalamin and cyanocobalamin.* Approximately 95 per cent of the radioactivity was associated with the areas corresponding to cyanocobalamin. A trace of radioactivity was associated with compound A in the liver. Similarly, reverse isotope dilution assay was carried out with muscle and again detectable radioactivity was associated with cyanocobalamin. These results justify the assumption that we were dealing with radioactive cobalamin convertible to cyanocobalamin in the liver and muscle.

TABLE III

Recovery of Vitamin B_{12} From Liver as Cyanocobalamin

Co^{60} Recovered (%)	Distribution Coefficient* Radioactivity	Color Reaction
95 ± 3.0	1.24	1.19
94 ± 2.0	1.26	1.23

* The distribution coefficient of crystalline cyanocobalamin was 1.21.

Preparation of Co^{60}-Labeled Food for Feeding to Volunteer Subjects

Normal subjects and patients with pernicious anemia were fed the Co^{60}-labeled vitamin B_{12}-containing liver and lean meat.

The liver was prepared as a well seasoned paté. It was either heated for 5 minutes at 100°C. in water or utilized raw. An amount calculated to provide 38 μg. of vitamin B_{12} was ingested as part of an otherwise nonvitamin B_{12}-containing meal. The muscle meat was served in the form of ground mutton patties prepared by wrapping in aluminum foil and by broiling the meat for 45 minutes at 340°F. In addition to the meat, each subject

* Courtesy of E. Alpert, Merck, Sharpe & Dohme, West Point, Pennsylvania; E. L. Smith, Glaxo Research Ltd., Greenford, Middlesex, England; and Dr. Konrad Bernhauer, Lehrstuhl Für Biochemie Der Technischen Hochschule, Stuttgart, Germany.

TABLE IV
Effect of Cooking* on Vitamin B_{12} Content of Food

Meat	Loss of Vitamin B_{12} (%)
Liver	8 ± 4
Muscle	27 ± 5

* Liver was immersed in boiling water for 5 minutes. Muscle was broiled in aluminum foil at 340°c. for 45 minutes.

received a slice of white bread, a green salad, pie and tea. The only significant source of vitamin B_{12} in the meal was the meat. There was at least a 4 hour interval between meals.

Absorption of Nonfood-Contained (Crystalline) Vitamin B_{12}

Co^{60} and Co^{58}-labeled vitamin B_{12} was obtained with a specific activity of approximately 1 μc. per μg. This was fed as an aqueous solution after appropriate dilution with nonradioactive vitamin B_{12}. The test feedings of labeled vitamin B_{12} were given with a nonvitamin B_{12}-containing meal to patients with pernicious anemia and to healthy subjects.

Determination of Vitamin B_{12} Absorption

The methods used have been described elsewhere in detail.[1,2,13] Briefly, a human whole body counter was used. Four hours after ingestion of the Co^{60}-labeled vitamin (as crystalline or food-bound vitamin) the subject was placed in the whole body counter, with the resultant value being taken as 100 per cent. The count was determined again ten to fourteen days later. Absorption was calculated from the formula:

$$\frac{\text{whole body count at ten to fourteen days}}{\text{whole body count at 4 hours}} \times 100 = \text{per cent absorbed}$$

In several subjects, it was possible to collect excreta during this time and to compare the results obtained with this technic for measuring absorption to those obtained with the whole body counter. The results obtained with the two methods were comparable.

As described earlier, there was a small amount (2 to 8 per cent) of Co^{60} present in the feedings of meat and liver which was not associated with vitamin B_{12}. This was considered to be not significantly retained at the end of fourteen days. Accordingly, corrections were made in the calculation of per cent absorption of vitamin B_{12}. This assumption is justifiable on the basis of reported studies.[6] We further confirmed these findings by feeding 100 μg. of $CoCl_2$ labeled with 0.5 μc. of Co^{60} to two subjects. At the end of one week less than 0.1 per cent of the radioactivity was present in the body.

RESULTS

The Effect of Cooking on Vitamin B_{12} Content of Food

Others have reported variable but definite degradation of vitamin B_{12} in food as a result of cooking.[14] Table IV indicates the loss of vitamin B_{12} activity, determined by assays using Euglena gracilis prior to and after cooking.

Five minute immersion of liver in boiling water did not result in substantial loss of vitamin B_{12} activity. However, when muscle meat was broiled, there was a loss of approximately 30 per cent of the vitamin B_{12} activity. This includes losses in the drippings as well as by inactivation. The value used for calculating absorption, therefore, is the amount of radioactivity which corresponded to the recovered cobalamin after cooking.

Absorption of Vitamin B_{12} from Lean Meat and Liver

Seven normal young men ingested ground mutton patties in amounts of 100, 200 and 300 gm. with the previously described accompanying food. This corresponded to 0.95, 3.03 and 5.11 μg. of vitamin B_{12}. The amount of Co^{60}-labeled vitamin B_{12} fed was identical in each group; however, the amount of vitamin B_{12} was

TABLE V
Absorption of Vitamin B_{12} from Cooked Lean Meat by Normal Young Subjects

Subject	Amount Fed (μg.)	Amount Absorbed %	Amount Absorbed μg.
J. M.	0.95	62	0.59
C. M.	0.95	56	0.53
J. C.	0.95	77	0.73
B. S.	3.03	89	2.70
A. M.	3.03	76	2.30
R. K.	5.11	63	3.20
R. L.	5.11	40	2.10

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varied by diluting the radioactive lamb meat with nonradioactive meat. The data suggest that the per cent absorption from the 3 μg. feeding was maximal and additional absorption from the succeeding 2 μg. was negligible (Table v). The values corresponded closely to results reported by others using crystalline vitamin B_{12}.[15,16] The increase in the absorption of vitamin B_{12} in amounts above approximately 2 to 3 μg. is negligible. The values are consistent with a block of active intrinsic factor mediated absorption at a level above 2.5 to 3 μg.

The liver paté was fed to six normal young men, four older persons who were hematologically normal and five patients with well established pernicious anemia. The results are summarized in Table VI. The five normal young men absorbed the most vitamin B_{12} (mean approximately 10 per cent) and the patients with pernicious anemia the least (1.8 per cent). The absorption in the older healthy subjects was intermediate and there was slight overlap of the absorption data for the older normal subjects with both the young subjects and the patients with pernicious anemia. Again the data are consistent with the concept of a block in intrinsic factor mediated absorption at about 3.0 μg. with absorption at higher levels of intake being "passive" or by "diffusion" and proportional to the amount ingested. The data also suggest a decrease in ability to absorb vitamin B_{12} which is age dependent, but the numbers are too few to be absolute in this regard.

TABLE VI
Absorption of Vitamin B_{12} from Liver*

Subjects	Amount Absorbed %	Amount Absorbed μg.
Normal Young Subjects		
R. L.	5.1	1.9
C. M.	5.5	2.1
P. P.	7.9	3.0
D. L.	10.3	3.9
W. S.	19.5	7.4
C. D.	16.5	6.3
	(9.1)	(4.1)
Normal Old Subjects		
E. W.	2.4	0.9
E. J.	3.8	1.4
V. M.	5.7	2.2
S. B.	6.0	2.3
	(4.5)	(1.71)
Patients with Pernicious Anemia		
S. N.	0.0	0.0
R. R.	0.8	0.3
J. C.	0.9	1.34
M. M.	2.7	1.03
B. V.	3.7	1.41
	(1.8)	(0.68)

* Liver paté containing 38 μg. vitamin B_{12} per feeding. Figures in parentheses represent the mean.

TABLE VII
Effect of Previous Feeding of Vitamin B_{12}* on Subsequent Absorption

Subject	Co^{60}-Vitamin B_{12} Absorbed %	Co^{60}-Vitamin B_{12} Absorbed μg.	Co^{58}-Vitamin B_{12} Absorbed %	Co^{58}-Vitamin B_{12} Absorbed μg.
D. D.	63	3.2	42	2.1
D. J.	55	2.8	39	2.0
A. R.	52	2.6	56	2.8
H. C.	51	2.6	38	1.9
	(55)	(2.8)	(44)	(2.2)

* 5 μg. of Co^{60}-vitamin B_{12} was fed approximately 5 hours prior to feeding of 5 μg. of Co^{58}-vitamin B_{12}. Figures in parentheses represent the mean.

Absorption of Vitamin B_{12} Fed at 5 Hour Intervals to the Same Subjects

In order to establish that previous intake of vitamin B_{12} (within 4 to 6 hours) did not interfere with subsequent absorption of the vitamin, four young subjects were given 5 μg. of Co^{60}-labeled vitamin B_{12} with a nonvitamin B_{12}-containing meal for breakfast. At lunch (5 hours later) they were given a similar feeding containing 5 μg. of Co^{58}-labeled vitamin B_{12}. As shown in Table VII, absorption from the two feedings was similar with only slightly less vitamin B_{12} being absorbed at the second feeding. There is neither long-lasting saturation of vitamin B_{12}-binding sites in the intestine nor any interference with transport across the intestinal wall. These data clearly show that if each meal contains as much as 3 to 5 μg. of vitamin B_{12}, then total absorption during a day from three meals could easily exceed 6 μg. Further, the amounts of crystalline vitamin absorbed by these subjects compare favorably with the data presented earlier (Table v) on absorption of vitamin B_{12} from food sources in normal young male volunteer subjects.

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COMMENTS

The studies described were undertaken to determine the amount of vitamin B_{12} absorbed daily from dietary sources. Our previous reports on loss rates of vitamin B_{12} indicated that this information was of critical importance in evaluating not only those studies but also in estimating minimal daily dietary requirements. Certain additional facts have emerged. These are the effect of cooking on the vitamin B_{12} content of meat, the possible effect of aging on the subject's ability to absorb vitamin B_{12} and the differences or similarities between absorption of crystalline cyanocobalamin fed with meals as opposed to native, food-bound vitamin B_{12}.

To assess the significance of the absorption studies recorded here, it is profitable to review, briefly, estimates of vitamin B_{12} losses from the body that we[1-3] and others[17,18] have obtained in recent years. The most relevant studies have employed whole body counting technics and Co^{60}-labeled vitamin B_{12} to assess loss rates of the vitamin. We have reported studies on both healthy subjects and patients with pernicious anemia following the injection of Co^{60}-labeled vitamin B_{12}. Observations in some subjects have extended over more than three years and are summarized in Table VIII. The patients with pernicious anemia were given variable doses of vitamin B_{12} ranging from 0.5 to 3 μg. per day by injection. Several of these patients had low body stores of vitamin B_{12} (the "low dose" group) whereas in the remainder body stores were probably intermediate between those in normal subjects and those in patients with pernicious anemia in relapse.[19-21] No significant differences in loss rate between the healthy subjects and the patients with pernicious anemia were detected. Within the dose range studied, the amount of vitamin B_{12} received by the patients did not appear to influence the rate of loss. The average daily loss of vitamin expressed as a rate constant in per cent was slightly greater than 0.1 per cent per day for both normal subjects and patients.

TABLE VIII
Turnover of Co^{60}-Labeled Vitamin B_{12}

Dietary Intake	Subjects (no.)	Daily Intake of Vitamin B_{12} (μg.)	Loss/Day (%) Mean	Range
Normal Subjects				
Normal	3		0.12	0.09–0.15
Patients with Pernicious Anemia				
Low dose	4	0.5–1.0	0.12	0.10–0.14
Moderate dose	7	1.5–4.0	0.14	0.11–0.17

These observations led us to propose that there is a fixed rate of loss of vitamin per day irrespective of the size of body stores. Put in other terms, this means that a normal person with 2 to 5 mg. in body stores would lose from 2 to 5 μg. per day, whereas a patient with body stores depleted to 500 μg. of total body vitamin B_{12} would lose approximately 0.5 μg. per day. Both estimates reflect a steady state situation with regard to vitamin B_{12} stores. As body vitamin B_{12} stores decline (if intake is limited or ceases) absolute amounts of the vitamin lost daily would decline in proportion although the *rate* of loss would remain constant.

These results and interpretations may seem surprising for two reasons. First, a high absolute loss of vitamin B_{12} in normal, well nourished persons is inferred. Secondly, the interpretations suggest that the concept of "normal" body stores of vitamin B_{12} is incorrect, and that above a certain critical level usual body stores probably depend more upon intake of the vitamin than on physiologic need for vitamin B_{12}. These views are not divergent from our understanding of the metabolism of other water-soluble vitamins, such as ascorbic acid or thiamine. At minimal intakes of such nutrients, urinary excretion approaches zero and conversely, as body stores become maximal (saturated) urinary loss increases. Recently Adams[22] has shown similar differences in the urinary losses following injections of vitamin B_{12} given to normal subjects and to patients with pernicious anemia. Further, if the considerations are viewed in terms of the wide variation in vitamin B_{12} intake around the world, the poor correlation of these data with the occurrence of megaloblastic anemia secondary to vitamin B_{12} lack,[1] and the solid evidence that amounts of vitamin B_{12} as minute as

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1 μg. or less per day are sufficient to maintain good health,[23,24] they seem reasonable.

The studies of two other groups differ with regard to daily body loss of vitamin B_{12}. Reizenstein and colleagues[17,25,26] have reported estimates of body loss of vitamin B_{12} derived from whole body counter studies, plasma clearance of vitamin B_{12} and fecal excretion studies. Because the radioactive tracer vitamin B_{12} was not considered to be in equilibrium with body pools of vitamin, compartmental analysis of the rate of loss of body vitamin B_{12} was undertaken using an analogue computer. The amount of vitamin lost daily by a healthy person with body stores of 4,000 μg. was estimated to be 1.3 μg. as opposed to our estimate of 4 μg. Heinrich,[18] using a whole body counter, also obtained a lower value for estimated daily loss of vitamin B_{12} from a normal subject.

It is not possible to reconcile the three studies completely. It is worth pointing out, however, that Reizenstein's three normal subjects were studied for only 250 days using the whole body counter, whereas our studies extended over one and a half to more than three years. Further, although nonequilibrium of tracers in body pools is an accepted concept,[27] in the model used for analogue simulation over 90 per cent of the tracer dose was in a single large compartment from which 90 per cent of the losses occurred. The contribution of losses from other pools was small and all losses can be essentially summed by a single rate constant derived from whole body counter studies.[17] The studies of Heinrich[18] are similar to ours in kind but differ in the rate of loss of tracer labeled-vitamin B_{12}. As measured in our laboratory, the loss of vitamin B_{12} is approximately two times as great as he has observed. Although dissimilar types of whole body counter systems were used, there is little reason to believe that the discrepancy in results reflects this fact. Resolution of the differing results in the various studies of body losses of vitamin B_{12} does not appear to be at hand.

There are, however, two compelling reasons for believing that our estimates of the total body loss of vitamin B_{12} are correct. Clinical observations indicate that in healthy persons in whom the dietary intake of vitamin B_{12} ceases, a lapse of from six months to eight years[23,28,29] is usually necessary before body stores of vitamin B_{12} are depleted and the megaloblastic state develops. Using the lower estimates of loss derived from other studies, this would not be expected to occur for ten to twenty years. Heinrich, based on his data, has actually calculated that vitamin B_{12} deficiency would occur in three to five years.[18] He used a linear function for loss rate to arrive at this estimate whereas his data as well as Reizenstein's and our own all show the loss of tracer to be an exponential function with time. Proper calculation of Heinrich's data would predict that vitamin B_{12} deficiency would not ensue for twelve and a half years following cessation of the dietary intake of vitamin B_{12}. If the larger value for loss rate than we have obtained is used, the period of time required for the deficiency to develop, starting from a reasonable level of body stores (2,000 to 5,000 μg.) ranges from three to eight years. This prediction is in accord with the clinical observations cited earlier.

The present studies also support the conclusion that 2 to 5 μg. is a more nearly correct estimate of loss. These studies on dietary absorption clearly indicate that if dietary vitamin B_{12} is divided into two or three reasonably spaced meals per day, and that if intake is of the order of 5 to 15 μg. per day,[30] the daily increment in body stores of vitamin would approximate 2 to 5 μg. or more. Unless there is a constant increase in body stores throughout life, such rates of gain of the vitamin must be balanced by similar rates of loss. The estimates of higher daily losses, which we obtained, more nearly coincide with the estimates for assimilation of dietary vitamin B_{12} than do the lower values.

Based on these data on absorption, body content and losses of vitamin B_{12} under varying circumstances, and estimates of minimal daily physiologic requirements for vitamin B_{12}, certain conclusions may be drawn concerning the minimal daily dietary requirement for vitamin B_{12}. It is clear that there is a level of body vitamin B_{12} below which clinical manifestations of disease occur. This is apparently on the order of 500 μg. of vitamin,[5] although an abso-

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lute minimum cannot be arrived at on the basis of present data. There seems to be no reason to believe that benefits (other than those of a ready supply available for periods of dietary restriction) attach to body levels of vitamin B_{12} *above* this critical level. Body levels of vitamin B_{12} would then seem to have more sociologic, ethnic and economic meaning, since they probably reflect the magnitude of the dietary intake of vitamin B_{12}, not the physiologic need. We believe that a subject with body content of a vitamin sufficient to prevent minimal functional evidence of a deficiency, such as macrocytosis in the instance of vitamin B_{12}, is in the zone of nutriture of "unsaturated but functionally unimpaired."[31] The only disadvantage such a person would have compared to persons with higher body levels would be the more rapid onset of symptoms of vitamin B_{12} deficiency if dietary intake ceased.

Considering that the body content of vitamin B_{12} above a certain minimal level is not a critical factor and that absolute body losses of vitamin B_{12} depend on the size of body stores, only two factors need to be known in order to set minimal daily dietary requirements: (1) the minimal amount of vitamin which will maintain health, and (2) the amount of vitamin in dietary sources which in the presence of normal absorption can be expected to supply that amount.

The physiologic maintenance dose for vitamin B_{12} given by injection to patients with pernicious anemia varies from approximately 0.5 to 1.0 μg. per day.[24] The term "physiologic maintenance dose" refers to that amount of vitamin which reverses all the currently recognized symptoms and signs of vitamin B_{12} deficiency. These amounts are of the same order as the minimum necessary to produce an initial *maximal* erythropoietic response. In a more recent report Sullivan and Herbert[32] reported that 0.1 μg. of injected vitamin B_{12} is the *minimal effective* daily dose of vitamin B_{12}. This latter concept is different from that of a minimal daily physiologic maintenance dose, since patients on 0.1 μg. did not exhibit maximal reticulocytosis and continued to have morphologic evidence of vitamin B_{12} deficiency, a result similar to that which we obtained with amounts of vitamin B_{12} less than 0.5 μg. Although 0.5 μg. of vitamin does result in reticulocytosis and gradual improvement in hemoglobin and hematocrit, many manifestations of vitamin B_{12} deficiency remain, including progression of neurologic disease. Viewed in these terms, the minimal daily adult requirement for vitamin B_{12} is certainly above 0.1 μg. and the earlier values of 0.5 to slightly greater than 1.0 μg. appear reasonable.

With regard to vitamin B_{12} absorption, the data cited here show that 60 to 80 per cent of a 1 to 2 μg. feeding of vitamin B_{12} in food is absorbed. We estimated earlier[24] that minimal daily dietary needs for this factor could be met by an amount of 0.6 to 1.2 μg. of vitamin in food assuming 80 per cent absorption. Since a somewhat lower absorption appears more correct, we would revise the estimated dietary requirement upward and believe that 0.9 to 1.8 μg. in food should provide a sufficient amount of the vitamin for the majority of people.

These considerations concerning minimal requirements of vitamin B_{12} and minimal dietary requirements are to be regarded as indicating "a 'floor' on vitamin B_{12} needs."[1] Such a dietary intake of vitamin B_{12} will maintain health and normal hemopoiesis in most subjects with a small body pool of the vitamin who are operating at an efficient physiologic economy. Storage bins filled to the "ceiling," if achieved, would be associated with a considerable loss of physiologic economy. A larger body pool results in a larger loss; to maintain maximal body stores would require higher levels of intake.

It is valid to recognize that to maintain body stores at the level of 2 to 5 mg., the dietary content of vitamin B_{12} must be sufficient to permit these accretions above the minimal needs and at the same time to meet the increasing losses which are associated with these larger stores. To fulfill these functions, the diet may need to afford 5 μg. or so of the vitamin per day. The evidence here presented indicates that such balances can be expected to occur on diets containing the usual quantities of vitamin B_{12} encountered in a wide variety of commonly consumed dietaries in the United States.[30]

Unlike some earlier reported data,[33–35] we could find no absorptive advantage conferred by vitamin B_{12} in food as opposed to crystalline vitamin B_{12}. Patients with pernicious anemia did not absorb dietary vitamin B_{12} from liver more efficiently than would have been expected, based on published data[15,16] on absorption of crystalline vitamin B_{12} by patients lacking intrinsic factor. This observation confirms those made earlier by Sullivan et al.[36]

SUMMARY AND CONCLUSIONS

Absorption of native vitamin B_{12} from food sources is quite comparable to absorption of the vitamin as crystalline vitamin B_{12}.

Normal adults eating a diet containing 5 to 15 μg. of vitamin B_{12} divided into three meals can and probably do absorb at least 5 μg. of the vitamin daily. Since there is an obligatory rate of loss of vitamin B_{12} which approximates 0.1 per cent daily of the body pool of the vitamin, this estimate of total daily absorption of vitamin B_{12} correlates well with the estimate of daily loss of body vitamin B_{12} assuming a body pool of vitamin B_{12} of 2 to 5 mg. Daily losses of vitamin B_{12}, or the body pool size of the vitamin in normal subjects eating food with a high content of the vitamin, do not reflect minimal daily dietary requirements. Estimates of minimal daily dietary requirement should be based on knowledge of the amount of vitamin which maintains good health in persons with depleted stores and to data related to turnover of body pools at low levels of body stores when physiologic economy is maximal.

A minimal daily dietary intake of vitamin B_{12} of 0.6 to 1.2 μg. is adequate to maintain health and normal hemopoiesis in normal subjects operating with low body stores. Any additional needs imposed by growth, hypermetabolic states or pregnancy are not known. It is valid to recognize the value of increased stores to meet periods of depletion. Accordingly, an estimate of 3 to 5 μg. of vitamin B_{12} as the dietary requirement for a population appears reasonable.

Severe heating of meat and meat products in preparation of food degrades vitamin B_{12}. Estimates of intake based on analysis of food prior to cooking are invalid. There may be an age-related decrease in the ability to absorb vitamin B_{12}.

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MALABSORPTION OF VITAMIN B_{12} IN MEGALOBLASTIC ANAEMIA AMONG AFRICANS AND INDIANS IN DURBAN

BY

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In a previous paper we attempted to classify megaloblastic anaemia as seen among Africans and Indians in Durban (ADAMS and HIFT, 1961). Pregnancy and poor nutrition appeared to be the main factors in causation while only one case out of 48 was regarded as being due to intestinal malabsorption on the basis of the vitamin A test (as a screen), and subsequent fat balance studies. Since then we have seen three further patients with malabsorption among 50 cases of megaloblastic anaemia. Twenty patients had impaired vitamin A absorption, and in 5 of the 10 in whom the test was repeated after treatment there was apparent improvement. In the same series of 50 a radioactive vitamin B_{12} absorption test was done in 11 cases and found to be abnormally low in five and doubtful in three. Similar results have been reported from India and Malaya (KAUL et al., 1959; TASKER, 1961).

In another paper one of us reported a striking increase in serum vitamin B_{12} levels in the majority of patients with megaloblastic anaemia treated with folic acid only, but could not decide whether this was due to temporary malabsorption of vitamin B_{12}, corrected by treatment with folic acid or to mobilization of vitamin B_{12} from the tissues (HIFT, 1963).

It therefore seemed important for us to study the role of malabsorption further. In this paper we report a preliminary study of vitamin B_{12} absorption in the common megaloblastic anaemias seen in Durban.

MATERIALS AND METHODS

We investigated 18 patients with megaloblastic anaemia, admitted consecutively, and diagnosed by examination of the bone marrow. Two were excluded because there was achlorhydria after maximal stimulation with histamine, and although there were atypical features, pernicious anaemia could not be ruled out. The remaining 16, consisting of 2 Indians and 14 Africans, all with free acid in the gastric juice, form the basis of this report.

The mean haemoglobin on admission was 3.6 g./100 ml. and the range 1.9 g. to 7.1 g./100 ml.

Ten patients with iron-deficiency anaemia, established by serum iron estimation and examination of the bone marrow for iron stores, were used as controls. Anaemia was comparable in degree (mean haemoglobin on admission 5.2 g./100 ml., range 3.4 g. to 8.1 g./100 ml.).

Serum vitamin B_{12} levels were determined by the method of HUTNER et al. (1956), and folic acid deficiency was assessed by the formiminoglutamic acid test as described by KNOWLES et al. (1960).

Radioactive vitamin B_{12} absorption was estimated by a modification of the method of SCHILLING (1953), using 5 ml. samples of urine in a well-crystal scintillation counter and 0.5 μc. Co^{60}-labelled

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vitamin B_{12}. In each case the test was performed within 3 days of admission. The patient was then treated with folic acid (30 mg. daily), and the test repeated at intervals of about 2 weeks until discharge, except in those in whom the first test was clearly normal. Most workers in this field would agree that a urinary excretion (without intrinsic factor) of more than 10 per cent. is normal, while less than 5 per cent. is abnormal.

An attempt has been made to separate different types of megaloblastic anaemia, but among our patients this is by no means easy, since most live on diets that would be regarded as poor by Western standards. Eight patients had recently been pregnant. The remaining eight, grouped together as "nutritional megaloblastic anaemia," included one who had cirrhosis of the liver and another who was a vegetarian and suffered from epilepsy, for which he had been taking anticonvulsants for many years. One patient had concomitant iron deficiency anaemia, but complicating diseases were otherwise absent in this series.

RESULTS

Serum vitamin B_{12} levels were above 100 $\mu\mu$g./ml. in 13 out of 14 patients, but more than half had less than 200 $\mu\mu$g./ml. The formiminoglutamic acid test was positive in all eight patients in whom it was done. Folic acid deficiency, therefore, appears to be the main cause of megaloblastic anaemia in this series, but vitamin B_{12} deficiency may co-exist in some patients.

The radioactive vitamin B_{12} excretion test ranged from 0 to 19.0 per cent., with a mean of 6.5 per cent. It was clearly normal (above 10 per cent.) in only three patients, while in eight it was in the doubtful range (5 per cent.-10 per cent.). In the remaining five patients there was vitamin B_{12} malabsorption (excretion less than 5 per cent.).

Although pernicious anaemia was ruled out in all patients by the presence of acid in the stomach, there remained a remote possibility of an acquired intrinsic factor deficiency as the main cause of their malabsorption. The excretion test was therefore repeated, with addition of intrinsic factor, in five patients in whom the clinical condition warranted a further delay before treatment with folic acid. In none of them did intrinsic factor produce a significant improvement in excretion.

Serial tests in those excreting less than 10 per cent. Co^{60}-labelled vitamin B_{12} showed well-marked rises in two patients (4.9 per cent. to 16.4 per cent., and 6.7 per cent. to 20.0 per cent.), both with megaloblastic anaemia related to pregnancy. They appear to demonstrate temporary malabsorption of vitamin B_{12}. Three patients with initial excretions of 0, 1.1 per cent. and 0.6 per cent. showed no significant rises after treatment with folic acid and must be regarded as cases of vitamin B_{12} malabsorption ; one of these had recently been pregnant. Minor changes occurred in some of the others with poor initial levels, but these are not commented upon further, since variations in the range from 5 per cent. to 10 per cent. are difficult to interpret.

In the control group of 10 patients with iron-deficiency anaemia, all but one excreted more than 10 per cent. of the test dose of radioactive vitamin B_{12}. The range was 9.1 per cent. to 33.3 per cent. and the mean 20.3 per cent.

DISCUSSION

In countries where dietary standards are poor it is difficult to classify cases of megaloblastic anaemia because the part played by malnutrition cannot readily be assessed. Using criteria we adopted previously (ADAMS and HIFT, 1961), we observed that 11 of these 16 patients had subsisted on diets poor in sources of folic acid, or vitamin B_{12}, or both ; while

in none could the diet be regarded as good. Megaloblastic anaemia therefore appeared to have a nutritional background in most of them, although there were other contributory factors such as recent pregnancy and cirrhosis. Serum vitamin B_{12} levels above 100 $\mu\mu$g./ml. in 13 out of 14 patients and the presence of formiminoglutamic acid in all eight patients tested suggest that folic acid deficiency was mainly responsible for the anaemia.

Hitherto we have observed that the incidence of malabsorption was low when judged on fat-balance studies, and we concluded that steatorrhoea was seldom a causative factor. What we have not known is whether there is malabsorption of other substances such as vitamin B_{12}. The work of METZ et al. (1960) in Johannesburg suggested that malabsorption of vitamin B_{12} does not play a significant role in the pathogenesis of megaloblastic anaemia associated with pregnancy, although five of their 30 patients excreted less than 5 per cent. of the test dose. The patients in our series, on the whole, absorbed radioactive vitamin B_{12} poorly (mean excretion 6.5 per cent.) when compared with a control group suffering from iron-deficiency anaemia (mean 20.3 per cent.), the difference between the means being statistically significant ($p < 0.001$). Low initial excretion levels and no rise after treatment showed that three patients suffered from vitamin B_{12} malabsorption.

Two other patients showed temporary malabsorption of vitamin B_{12}, corrected by treatment. Judging from the results obtained in our control group, anaemia *per se* does not appear to interfere with absorption of vitamin B_{12}. Despite the experimental work of HSU et al. (1962) on rats, demonstrating that induced folic acid-deficiency does not disturb vitamin B_{12} absorption, the results in these two patients and the rises we have previously observed in serum vitamin B_{12} levels in some patients with megaloblastic anaemia treated only with folic acid (HIFT, 1963) suggest that folic acid-deficiency may cause malabsorption of vitamin B_{12}.

SUMMARY

1) The absorption of radioactive vitamin B_{12} was investigated in 16 African and Indian patients with megaloblastic anaemia due to folic acid deficiency.

2) The mean urinary excretion (6.5 per cent.) was poor compared with a control group of patients with iron-deficiency anaemia (mean 20.3 per cent.).

3) Serial tests showed that definite vitamin B_{12} malabsorption was persistent in three patients, while in two others it was temporary, corrected by treatment.

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Methods in Enzymology

[203] Identification and Investigation of Cobalamins and Cobamide Coenzymes by Nuclear Magnetic Resonance and Electron Paramagnetic Resonance Spectroscopy

By H. A. O. Hill, J. M. Pratt, and R. J. P. Williams

The applicability of resonance methods to the study of biological systems has been markedly increased by recent advances in instrumentation. This has been most noticeable for nuclear magnetic resonance (NMR) spectroscopy. The high molecular weights and low solubility of many of the molecules involved in biological systems cause problems of sensitivity, resolution, or assignment. Although cobalamins and cobinamides may now be considered "small" molecules by comparison with some of the biological macromolecules that have been profitably investigated,[1] there remain problems of assignment that have not been overcome. The study of the

[1] For recent reviews see: C. D. Jardetzky and O. Jardetzky, *Methods Biochem. Analy.* **9**, 235 (1962); A. Kowalsky and M. Cohn, *Ann. Rev. Biochem.* **33**, 481 (1964); A. Kowalsky and M. Cohn, *in* "Magnetic Resonance in Biology" (B. Malstrom and T. Vanngard, eds.). Macmillan (Pergamon), London, 1967.

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coenzyme forms in the presence of the apoenzymes and substrates by NMR and electron paramagnetic resonance (EPR) spectroscopy has only just begun. It is therefore considered opportune to discuss the experimental problems involved in the study of simple cobalamins and cobinamides and the results obtained.

Nuclear Magnetic Resonance Spectroscopy

The angular momentum associated with the spin of a given nucleus is determined by a quantum number I, which can be integral, half-integral, or zero. Although it should be possible to study the $^{59}Co(I = 7/2)$ and $^{13}C(I = \frac{1}{2})$ NMR spectroscopy of cobalamins and cobinamides, we shall consider only that of the proton, for which $I = \frac{1}{2}$.

The angular momentum about a defined direction, e.g., that of the applied field H, is given by $m_I h/2\pi$ where h is Planck's constant and m_I is the spin quantum number, having values $I, I - 1, \ldots, -I + 1, -I$. The component of the nuclear magnetic moment along the defined direction (taken here as the z-axis coincident with the applied field) is given by

$$\mu_z = \gamma m_I h/2\pi = g_N \beta_N m_I \tag{1}$$

where γ is the magnetogyric ratio (26,753 radians sec^{-1} $gauss^{-1}$ for ^1H) g_N is a dimensionless constant, the nuclear g-factor (5.585 for ^1H) and β_N is the nuclear magneton given by $eh/4\,mc$ where e is the electronic charge, m is the mass of the proton, and c is the velocity of light. The interaction of the magnetic moment with the applied field, H, gives rise to two nuclear spin states whose energies are given by

$$E = -\mu_z H = \pm\tfrac{1}{2} g_N \beta_N H \tag{2}$$

where $m_I = \pm\frac{1}{2}$.

We may assume that both spin states will be populated and at thermal equilibrium there will be a slight excess in the lower energy level. It is possible to induce transitions between the two nuclear spin levels by applying an oscillating electromagnetic field whose magnetic vector is perpendicular to the applied field and whose frequency satisfies the resonance condition:

$$h\nu = g_N \beta_N H \tag{3}$$

Normally the field at the nucleus differs from the applied field because the electrons in the atom or molecule set up secondary magnetic fields at the nucleus. Therefore, the effective field at the nucleus H_{eff} differs from that applied and this effect is described by Eq. (4).

$$H_{eff} = H(1 - \sigma) \tag{4}$$

where σ is a screening constant. The frequency of the oscillating magnetic field is usually held constant, and the applied field is varied. Therefore if

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different nuclei have different electronic constants, σ_i, reflecting different electronic environments, resonance will occur at different applied fields. Experimentally we describe these effects by reference to the resonance condition of an arbitrarily chosen standard substance, in terms of the *chemical shift* δ. This dimensionless parameter is given by Eq. (5)

$$\delta_i = \left(\frac{\nu_i - \nu_r}{\nu_0}\right) 10^6 \tag{5}$$

where $\nu_i - \nu_r$ is the chemical shift difference between the sample and the reference in frequency units (cycles sec^{-1} or Hz) and ν_0 is the applied radio-frequency. In this chapter we will use the *tau* scale which assigns to tetramethylsilane $(CH_3)_4Si$ a chemical shift, $\tau = 10$ and those of other resonances are given by $\tau_i = 10 - \delta_i$.

The absorption of radiation can be detected only if there is a population difference between the two spin states. If we assume a normal Boltzmann distribution then we have:

$$\frac{N_{\text{ground}}}{N_{\text{excited}}} = e^{\Delta E/kT} = e^{g_N\beta_N H/kT} \simeq 1 + g_N\beta_N H/kT \tag{6}$$

where k is Boltzmann's constant, and T is the temperature, °K. The populations of the two spin states differ by only one part in 10^6 when $H = 10{,}000$ gauss. The difference in population increases with H, and therefore it is usually advantageous to work at high fields not only to "spread-out" the energy levels, thereby aiding assignment, but also to increase the sensitivity.

However there are two additional factors which must be considered. If we excite nuclei to the spin state of higher energy, the difference in population between the two levels must decrease. Therefore we might expect the resonance to disappear on prolonged irradiation. Obviously there must be some mechanism by which the excited state can be depopulated. This takes place by a nonradiative process described by spin-lattice relaxation in which the nuclear spin system is coupled to the thermal motions of the sample. The rate of absorption of energy can be described by Eq. (7)

$$\begin{aligned} \frac{dE}{dt} &= (N_{\text{ground}} - N_{\text{excited}})\mathbf{P}E \\ &= N_{\text{ground}}E\,\frac{\mathbf{P}}{1 + 2\mathbf{P}T_1} \end{aligned} \tag{7}$$

where $\mathbf{P}$ is the probability of transition between the two spin states and T_1 is the *spin-lattice relaxation time* which characterizes the relaxation process. Thus we see that the signal strength is proportional to the number of nuclei in the ground state and the energy separation. This will be so

provided $2PT_1 \ll 1$. If T_1 is long, i.e., the relaxation process is slow, the population difference between the two states will decrease and the signal can easily be "saturated." Since **P** is proportional to the square of the radiofrequency field amplitude, one usually keeps the power low to avoid saturation.

Changes in the lifetime of the excited spin state will lead to changes in the observed line width since the uncertainty in energy and the uncertainty in time are related by Heisenberg's uncertainty principle. There are also other processes which lead to a change in line width by varying the relative energies of the spin states rather than their lifetimes. These are described by a *spin-spin relaxation time* T_2. Both T_1 and T_2 depend on the movement of the molecules in solution. If the movement is rapid and the time taken for the molecule to complete, for example, a rotation is short and is described by a *correlation time* τ_c, then it can be shown that $T_1 \simeq T_2$. τ_c is proportional to the viscosity of the solution and inversely proportional to the temperature. An increase in τ_c will lead to an increase in T_1 and consequently an increase in the line width.

Another factor which is important in determining the NMR spectrum of a molecule, is the coupling between the nuclear spins. It is found that the interaction between the magnetic moment of a nucleus with that of a neighboring electron leads to a change in the field at an adjacent magnetic nucleus. If there are a number of relative orientations of the two nuclear magnetic moments, a number of energy levels arise causing fine structure in the spectrum. This is characterized by a *spin-spin coupling constant J* measured in cycles sec^{-1} or Hz. It is independent of field strength.

Experimental Problems

There are two principal experimental problems; sensitivity and resolution. The former may arise because only small amounts of sample are available or difficulties in preparing a sufficiently concentrated solution; the latter because of overlapping resonances, protons with similar chemical shifts, broadened lines due to spin-spin coupling or high viscosity with a consequent slow correlation time for molecular motion.

Solubility. Cobalamins and cobinamides dissolve in protic and aprotic polar solvents. The solubility is presumably governed by hydrogen bonding of the solvent to the peripheral amide groups and by the charge on the complex. Most cobalamins and cobinamides give 0.1 *M* solutions in water at room temperature, the principal exception being cyanocobalamin, which is much less soluble. Methanol, dimethyl sulfoxide, *N,N'*-dimethyl formamide, and trifluoroacetic acid are useful solvents.

Signal-to-Noise (S:N) Ratio. This can be increased by increasing the applied magnetic field. Therefore, although some recently developed 60

MHz instruments have high specifications, it is preferable to use a 100 MHz or indeed an instrument which has a superconducting magnet and operates at, e.g., 220 MHz. The latter are not inexpensive and a simpler method of increasing the S:N ratio, provided the sample does not change with time, is to accumulate the spectrum using a digital computer. Because the increase in the signal is proportional to $\sqrt{n}$, the number of spectra accumulated, whereas that of the noise is proportional to $\sqrt{n}$, the resultant increase is $\sqrt{n}$. Normally 100 accumulations, giving an increase in $S:N$ of 10 is optimal. A simpler method, which is applicable to cobalamins and cobinamides, is to use a very slow scan speed with a slow response time.

Though broad resonances cause problems of assignment, they do make it possible to work with a lower attenuation of the RF power than is customary for smaller organic molecules.

Resolution. The line width of a resonance depends on T_1 and T_2. In studying the ^{1}H NMR spectra of cobalamins and cobinamides, the effect of the viscosity on the correlation times is perhaps the most important factor. The viscosity increases with increasing concentration; consequently there is an optimum concentration, further increase in the concentration *decreasing* the signal *height* (but not the area) due to the increased line width. The viscosity can be lowered by using trifluoroacetic acid as solvent, but since it reacts slowly with the compounds it is not recommended. Although D_2O is the most convenient and the least expensive of the deuterated solvents, and indeed the most relevant to the study of biological materials, the solutions are relatively viscous and methanol-d_4, CD_3OD, is the preferred solvent.

Increasing the temperature, although it decreases the S:N ratio, reduces the viscosity. The effect is quite marked as shown[2] in Fig. 1. It is therefore useful to work at the highest temperature compatible with chemical stability.

The most useful method of reducing the overlap of resonances is to increase the field. This is where work at 220 MHz is most valuable, as shown[2] in Fig. 2. Note the increased resolution even though the 220 MHz spectrum was measured at a lower temperature.

Assignment of Spectra

The use of D_2O as solvent effectively blocks out that portion of the spectrum between 4 and 6 τ. When instruments are used that "lock-on" to a control signal, it is convenient to use the residual HOD peak for this purpose and sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as

[2] S. A. Cockle, H. A. O. Hill, B. E. Mann, J. M. Pratt, and R. J. P. Williams, unpublished results.

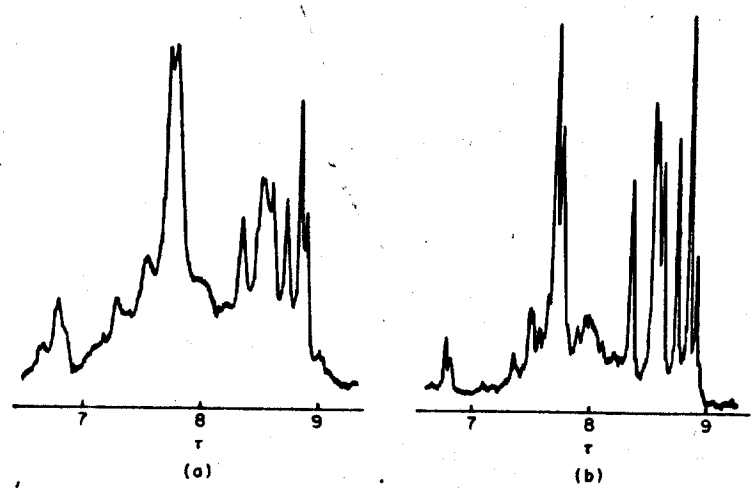

FIG. 1. ^{1}H NMR spectrum of dicyanocobinamide in $(CD_3)_2SO$ measured at 100 MHz and (a) at 31° and (b) 85°.

internal standard. Using methanol-d_4, the residual CD_3OH, or the internal standard (TMS), can be used as the control signal.

The assignments of many of the resonances observed in the spectra of various cobalamins and cobinamides are given[2,3] in Tables I–III. These

TABLE I
LOW-FIELD ^{1}H NMR SPECTRA OF THE COBALAMINS

Cobalamin	Benzimidazole C-2 (τ)	Benzimidazole C-4 (τ)	Benzimidazole C-7 (τ)	Ribose C-1 (τ)	Corrin C-10 (τ)	Adenine C-2 (τ)	Adenine C-8 (τ)
Aquo-[a]	(2.75	3.47	3.54)	3.72	3.72	—	—
Hydroxo-[a]	(2.88	3.52	3.25)	3.71	3.92	—	—
Cyano-[b]	2.76	3.43	2.89	3.72	3.96	—	—
Vinyl-[a]	2.77	3.58	2.88	3.71	4.05	—	—
Methyl-[a]	3.03	3.71	2.80	3.71	4.11	—	—
Ethyl-[a]	2.99	3.74	2.84	3.74	3.95	—	—
C_5-Deoxyadenosyl-[a]	3.03	3.73	2.81	3.73	4.04	1.77	1.97
β-Hydroxyethyl-[c]	3.05	3.72	2.83	3.72	3.96	—	—

[a] ~0.1 *M* in D_2O at 30° measured at 100 MHz.
[b] ~0.02 *M* in CD_3OD at 30° measured at 100 MHz.
[c] ~0.1 *M* in D_2O at 18° measured at 220 MHz.

[3] H. A. O. Hill, B. E. Mann, J. M. Pratt, and R. J. P. Williams, *J. Chem. Soc. Ser. A* p. 564 (1968).

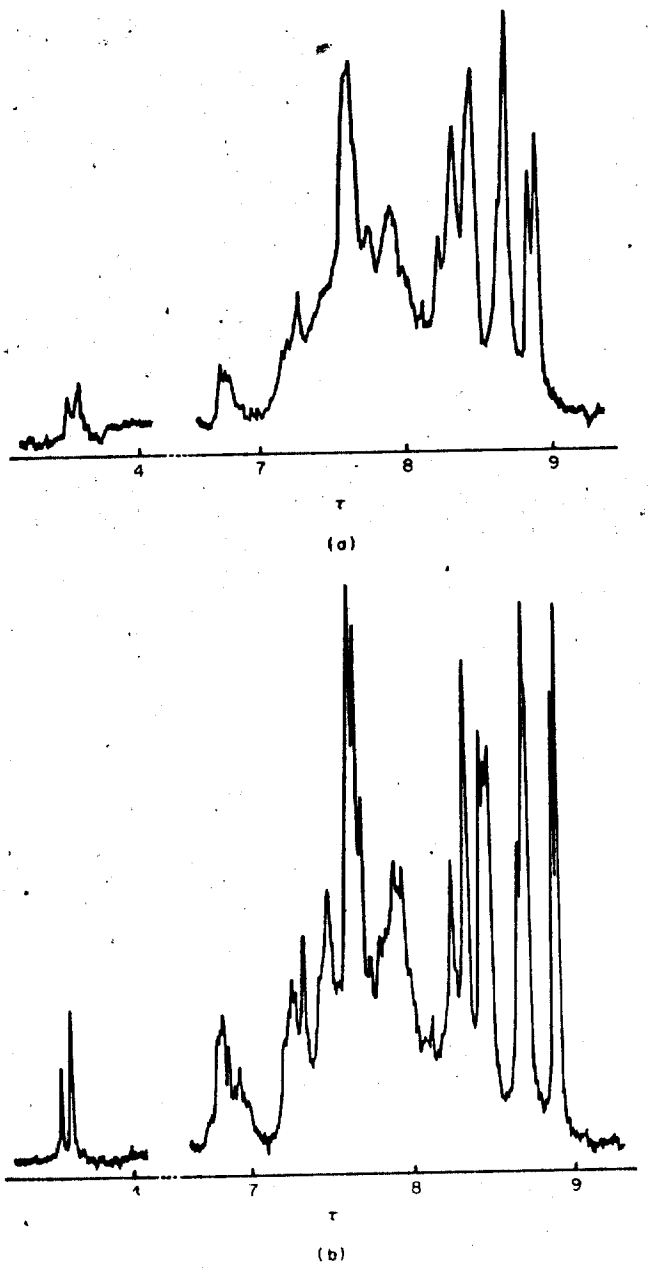

FIG. 2. ^1H NMR spectrum of cyanoaquocobinamide in D_2O measured at (a) 100 MHz and 31° and (b) 220 MHz and 18°.

TABLE II
HIGH-FIELD ^{1}H NMR SPECTRA OF THE COBALAMINS

Cobalamin	C-5 and C-6 methyls (τ)	C-5 and C-15 methyls (τ)	Corrin unassigned resonances (τ)	CH_3 of aminopropanol (τ)	Corrin C-1 (τ)	Cobalt-alkyl (τ)
Aquo-[a]	7.28, 7.33	7.68, 7.73	8.03, 8.47, 8.59	8.67, 8.74	9.45	—
Hydroxo-[a]	7.37	7.73	8.13, 8.59, 8.70	8.70?	9.55	—
Cyano-[b]	7.42	7.71	8.10, 8.61, 8.80	?	9.60	—
Vinyl-[a]	7.43	7.71	8.14, 8.71, 8.99	8.71	9.51	—
Methyl-[a]	7.55	7.80	8.22, 8.67	?	9.53	10.14
Methyl-[a]	7.62	7.71	—	—	—	10.19
Ethyl-[a]	7.48	7.77	8.02, 8.19, 8.58, 8.92	8.69	9.45	10.61
β-Hydroxyethyl-[d]	7.44, 7.46	7.74	7.28, 7.35, 7.91, 8.16, 8.53, 8.56, 8.84, 9.03	8.75, 8.78	9.48	9.38
Ethyl-[c]	7.63	7.79	7.95, 8.19, 8.39, 8.65	—	—	—
C_5-Deoxyadenosyl[d]	7.49	7.75	7.30, 7.88, 8.25, 8.58, 8.63, 9.10	8.76, 8.81	9.14?	11.12
C_5-Deoxyadenosyl[c]	7.61	7.72	7.22, 8.18, 8.34, 8.52, 8.62, 8.79, 9.07, 9.18	8.73, 8.77	9.47	9.40
				8.73	—	—

[a] ~0.1 *M* in D_2O at 30° measured at 100 MHz.
[b] ~0.02 *M* in CD_3OD at 30° measured at 100 MHz.
[c] ~0.1 *M* in D_2O, pD ~ 1, at 30° measured at 100 MHz.
[d] ~0.1 *M* in D_2O at 18° measured at 220 MHz.

TABLE III
^{1}H NMR SPECTRA OF COBINAMIDES

Cobinamide	C-10 (τ)	C-5 and C-15 methyls (τ)	Corrin unassigned resonances (τ)	CH_2 of aminopropanol (τ)	Cobalt-alkyl (τ)
Dicyano-[a]	4.13	7.71, 7.75	8.32, 8.44, 8.52, 8.70, 8.82	?	—
Vinyl cyano-[b]	4.58	7.82	8.24, 8.50, 8.73, 9.04	8.92, 8.97	—
Cyanoaquo-[c]	3.47, 3.53	7.60, 7.64	7.68, 7.89, 8.24, 8.33, 8.39, 8.46, 8.64, 8.68	8.86, 8.89	—
Methyl-[a]	3.20	7.52, 7.57	8.00, 8.16, 8.41, 8.56, 8.78	8.83, 8.90	10.24
Ethyl-[a]	3.04	7.50, 7.60	7.09, 7.96, 8.10, 8.38, 8.46, 8.53, 9.04	8.81, 8.87	9.70
Isopropyl-[d]	3.03	7.44, 7.50	8.13, 8.31, 8.49, 8.69, 8.89, 9.14	8.83, 8.89	10.70
Vinyl-[a]	4.05	7.50, 7.58	8.17, 8.40, 8.46, 8.63, 8.85, 9.05	8.80, 8.85	10.94

[a] ~0.1 *M* in D_2O at 30° measured at 100 MHz.
[b] ~0.1 *M* in $(CD_3)_2SO$ at 30° measured at 100 MHz.
[c] ~0.1 *M* in D_2O at 18° measured at 220 MHz.
[d] ~0.1 *M* in CD_3OD at 30° measured at 100 MHz.

were arrived at by the normal methods of assignment; comparison with similar compounds, deuterium substitution, calculation of neighboring-group effects, and correlation with other physical properties. The more significant groups in the molecules are discussed below. It has not yet been possible to assign many of the resonances in the 7–9 τ region; the CH_2CONH_2 and C—C— H resonances are probably in the 7–8 τ region, and the CH_2—CH_2—$CONH_2$ and methyl groups in the 8–9 τ region.

The C-10 Hydrogen. This can be easily assigned. Only one resonance is observed to low-field of HOD in the ^1H NMR spectra of most cobinamides, and it has τ values 3–4. It exchanges in acidic D_2O[3] and CF_3COOD[4] and is assigned to the C-10 hydrogen. It is also absent[5] in the C-10 chlorinated derivative. In the spectrum (Fig. 2) of cyanoaquocobinamide, two resonances are observed at 3.47 and 3.53 τ with an intensity ratio 4:6 and a total area equivalent to one hydrogen. These are assigned[3] to the C-10 hydrogens of the two possible positional isomers[6] of cyanoaquocobinamide, i.e., one with the cyanide on the same side as the C-19 hydrogen, the other with the cyanide on the opposite side. The assignment of the resonance at 4.04 τ to the C-10 hydrogen of 5,6-dimethylbenziminazolyl cobamide (DBC) coenzyme ($C_{5'}$-deoxyadenosylcobalamin) was confirmed by deuterium exchange.

5,6-Dimethylbenzimidazole Hydrogens. In the spectra of cobalamins with the nucleotide coordinated, there are three additional resonances in the low-field region of the spectrum. For example, in that of methylcobalamin, they occur at 3.03, 3.71, and 2.80 τ. They are assigned to the C-2, C-4, and C-7 hydrogens of the benzimidazole. The shift of the resonance due to the C-4 hydrogen to higher field than that of the C-7 may be caused by the proximity of the former to the π-electron system of the corrin ring or the cobalt. When the base is uncoordinated and protonated, the chemical shifts are similar to that of 5,6-dimethylbenzimidazole in acid.

The C-5 and C-6 methyl hydrogens give rise to resonances at 7.60 τ in the spectra of base-off cobalamins. When the base is coordinated the assignment is not always straightforward because of the similar chemical shift of the C-5 and C-15 methyl hydrogens of the corrin ring.

C-1′ Hydrogen of Ribose. In aqueous solution the spectra of the cobalamins have a resonance due to a single hydrogen at 3.72 τ. By comparison with the ^1H NMR spectra[7] of α- and β-5,6-dimethyl-1-α-D-2-deoxyribofuranosylbenzimidazole, this has been assigned to the C-1′ hydrogen of the ribose.

[4] R. Bonnett and D. G. Redman, *Proc. Roy. Soc.* (*London*) **288A**, 342, (1965).
[5] H. A. O. Hill, J. M. Pratt, and R. J. P. Williams, *J. Chem. Soc.* p. 2895 (1965).
[6] R. A. Firth, H. A. O. Hill, J. M. Pratt, and R. G. Thorp, *J. Chem. Soc. Ser. A* p. 453 (1968).
[7] C. P. Whittle and R. K. Robins, *J. Am. Chem. Soc.* **87**, 4940 (1965).

The $C_{5'}$-Deoxyadenosine of DBC Coenzyme. The spectrum of free adenosine has resonances at 1.18 τ (C-2), 1.27 τ (C-8), and 3.47 τ (C-1′). Based on this spectrum, the resonances at low field in that of DBC coenzyme (Fig. 3), in addition to those already discussed, may be assigned as follows; C-2, 1.77 τ; C-8, 1.97 τ, and C-1′ of the ribose at 4.34 τ.

Cobalt-Alkyl Resonances. The hydrogens of coordinated alkyl groups often give rise to resonances to high field of those of "normal" alkyl groups. Thus it is easy to identify the coordinated methyl group in methylcobalamin and methylcobinamide. However, with other alkyl ligands, identification of all cobalt-alkyl resonances is not so easy. In the spectrum of ethylcobalamin, the resonance at 10.61 τ (triplet) corresponding to three hydrogens, can be immediately assigned to the Co—CH_2—CH_3, but that of the Co—CH_2—CH_3 cannot be so readily identified. However, in the spectrum of ethylcobinamide, the resonance of the Co—CH_2—CH_3 is shifted to 11.2 τ and a poorly resolved quartet is observed at 9.70 τ, which is presumably that of the Co—CH_2—CH_3. The spectrum of isopropylcobinamide has *two* doublets at 10.70 τ and 10.94 τ, each corresponding to three hydrogens, assignable to the two methyl groups. The nonequivalence of the methyl groups suggests the absence of free rotation of the coordinated alkyl with a consequent unequal population of the various possible rotamers. Presumably rotation of the coordinated adenosine will also be hindered in DBC coenzyme, and we would not therefore expect the $C_{5'}$ hydrogens to be equivalent. The resonance, corresponding to one hydrogen

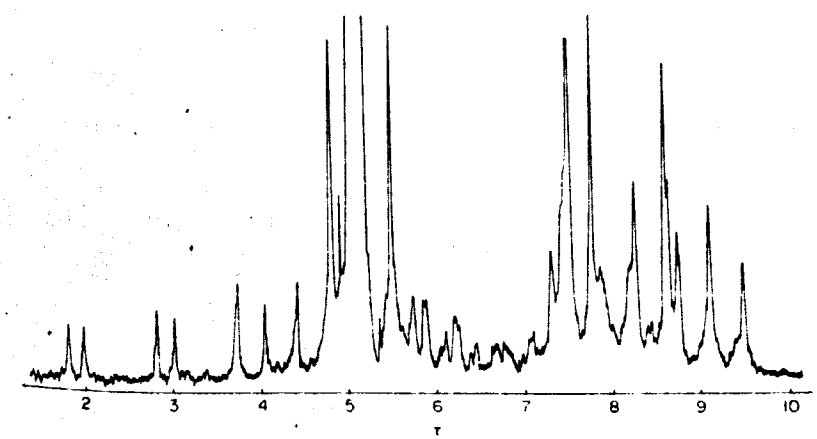

FIG. 3. ^1H NMR spectrum of DBC coenzyme in D_2O at 220 MHz and 18°. (The resonances between 4.7 and 5.5 τ are due mainly to residual HDO.)

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1232

which is observed in the spectra of DBC and β-hydroxyethylcobalamin at 9.4 τ, could be one C-5′ hydrogen. The other may be about 9.1 τ.

The C-5 and C-15 Methyl Groups. The assignment of the resonances due to these hydrogens in the spectra of cobinamides is trivial. They are prominent at ~7.5 τ. We have already stated that in the spectra of base-off cobalamins the C-5 and C-6 methyl hydrogens of the 5,6-dimethylbenzimidazole show a resonance at 7.60 τ; the other resonance at ~7.5 τ is assigned to the C-5 and C-15 methyl hydrogens.

C-1 Methyl Group. All the spectra of base-on cobalamins show a resonance at ~9.5 τ corresponding to three hydrogens. In the spectra of the cobinamides and base-off cobalamins it is absent. The X-ray determinations[8,9] of the structures of cyanocobalamin and DBC coenzyme show that the C-1 methyl group lies above the plane of the coordinated 5,6-dimethylbenziminazole. It is well known[10] that hydrogens which lie above and "inside" a ring of delocalized electrons are shifted to high-field by the ring current. Calculations have shown[2] that the C-1 methyl hydrogens will be shifted to high-field when the 5,6-dimethylbenziminazole is coordinated to the cobalt.

Temperature-Dependent Chemical Shifts—Five-Coordinate Species. The ^{1}H NMR spectra of some cobinamides are markedly temperature dependent in certain solvents.[11] For example, in Fig. 4, the temperature dependence of the chemical shifts of the CoCH_3 and C-10 hydrogen of methylcobinamide in D_2O (0–100°C) and CD_3OD (−60° to 50°C) are given in A and B together with that of vinylcobinamide and others. We have concluded that there are three classes of cobinamides; (a) Those with relatively "weak" ligands, e.g., H_2O, NH_3, whose ^{1}H NMR and electronic absorption spectra are insensitive to temperature changes. (b) Those with one very strong ligand, e.g., C_2H_5, whose ^{1}H NMR and absorption spectra are virtually temperature independent. (c) Those with ligands of intermediate strength, e.g., —CH=CH_2. With these ligands there is a marked dependence of the ^{1}H NMR and electronic absorption spectra with temperature suggesting the presence of two complexes in equilibrium.

For various reasons we consider the two complexes in class (2) to be an equilibrium mixture of a six-coordinate cobalt complex as in class (a) and a complex in which the coordination number of the cobalt approaches more closely five as in class (b).

[8] D. C. Hodgkin, J. Lindsey, M. Mackay, and K. N. Trueblood, *Proc. Roy. Soc. (London)* **266A**, 494 (1962).
[9] P. G. Lenhert, *Proc. Roy. Soc. (London)* **303A**, 45 (1968).
[10] See J. Musher, *in* "Advances in Magnetic Resonance" (J. S. Waugh, ed.), Vol. 2, p. 177. Academic Press, New York, 1966.
[11] R. A. Firth, H. A. O. Hill, B. E. Mann, J. M. Pratt, R. G. Thorp, and R. J. P. Williams, *J. Chem. Soc. Ser. A* p. 2419 (1968).

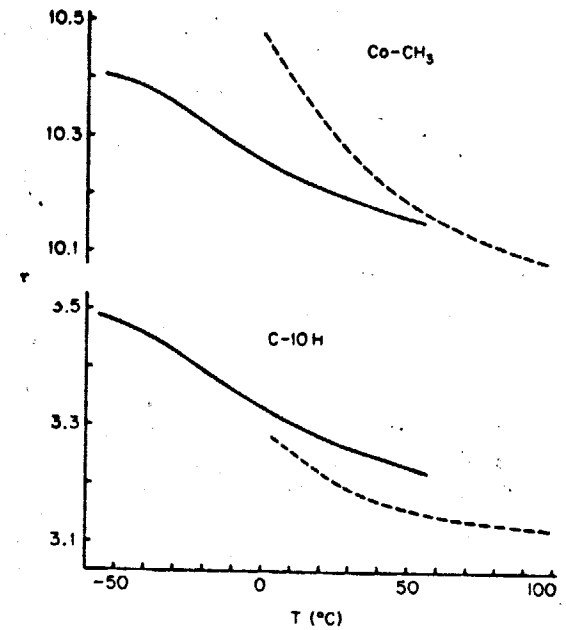

FIG. 4. The temperature dependence of the ^{1}H NMR spectra of the CoCH_3 and the C-10 H of methylcobinamide; in D_2O (- - - - -) and CD_3OD (——).

Transmission of Electronic Effects. There is a correlation[12] between the β-band, which is the 0-1 vibrational component of the first electronic transition, and the ^{1}H chemical shift of the C-10 hydrogen as shown in Fig. 5. The latter cannot be a general relationship, but is expected here, as the first excited state of corrin has[13] a node at C-10 and therefore the energy of the transition reflects changes in electron density at C-10 in the ground state, which presumably governs the C-10 H chemical shift.

The Application of NMR Spectroscopy to B_{12}-Dependent Enzymes

A high resolution study of most B_{12}-dependent enzymes will require considerable improvement in sensitivity if structural information is required. The long correlation time for the large molecule will also broaden the resonances. However, it may still be possible to identify resonances due to the coenzyme, particularly those in a region of the spectrum where few others occur, e.g., those of the 2-, 4-, and 7-hydrogens of 5,6-dimethylbenzimidazole, or if the resonances are very prominent, e.g., the C-5,

[12] R. A. Firth, H. A. O. Hill, J. M. Pratt, R. G. Thorp, and R. J. P. Williams, *J. Chem. Soc. Ser. A* p. 2428 (1968).
[13] P. Day, *Coordination Chem. Rev.* **2**, 109 (1967).

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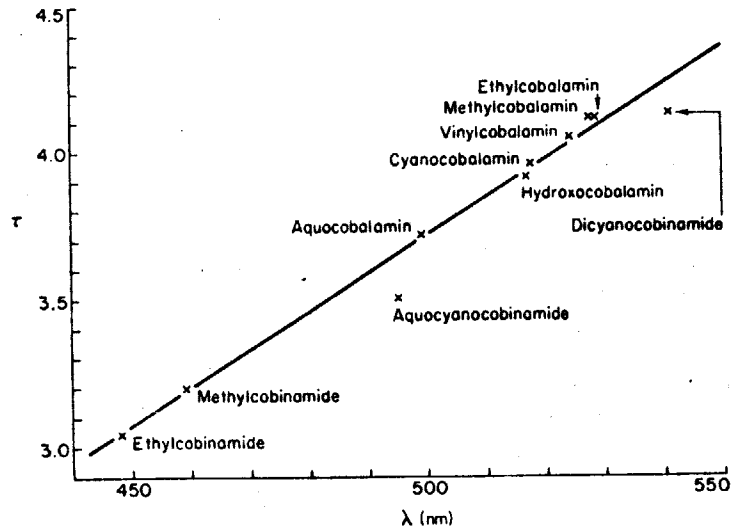

FIG. 5. The relationship between the chemical shift of the C-10 H (τ) and the wavelength of the β band (nm).

C-15 methyl resonances at 7.2 τ. It might then be possible to decide, for example, whether the nucleotide is coordinated or whether the cobalt-carbon bond is broken. An ^{1}H NMR study of the coenzyme isolated after reactions using deuterated solvents or substrates should indicate hydrogens which had exchanged, possible candidates being those of carbon ligands or, less likely, at C-10.

The magnetic moment of the single unpaired electron of cobalt(II) derivatives may have a large effect on the chemical shift or the line width of resonances due to adjacent hydrogens. If cobalt(II) derivatives are intermediates, the effect on the ^{1}H NMR of substrates or even water may yield valuable information.

Electron Paramagnetic Resonance Spectroscopy

It is customary to separate the total angular momentum of an electron into an orbital and a spin contribution. In many molecules there is no resultant orbital angular momentum; it is said to be "quenched," and the angular momentum properties are well described by a spin-quantum number S. In molecules containing heavier atoms, including most transition-metal complexes, we must allow for the error in our original assumption which separated orbital and spin angular momentum by including an additional contribution to the angular momentum from spin-orbit coupling.

The magnetic moment, μ, of the electron in the absence of an orbital contribution is given by $\mu = -g\beta S$, where g is the electronic g-factor, β is the Bohr magneton and equal to $eh/4\pi\ mc$, where e is the electronic charge, h is Planck's constant, m is the mass of the electron, and c is the velocity of light. The interaction of the magnetic moment of the electron with an applied field H will give rise to a number of energy levels given by

$$E = g\beta H m_s \tag{8}$$

where m_s gives the value of the angular momentum, in units of $h/2\pi$, about a defined axis (that coincident with the applied field). It can have values $S, S-1, \ldots -S+1, -S$. For a single electron $S = \frac{1}{2}$, therefore, two energy levels arise as shown in Fig. 6. Transitions can be induced between the two levels by an oscillating electromagnetic field with the magnetic vector perpendicular to H and with a frequency ν which satisfies the resonance condition

$$h\nu = g\beta H \tag{9}$$

For a free electron $g = 2.0023$, but most transition metal complexes deviate from this because of residual orbital contributions.

If the unpaired electron is localized, or partly localized, on an atom

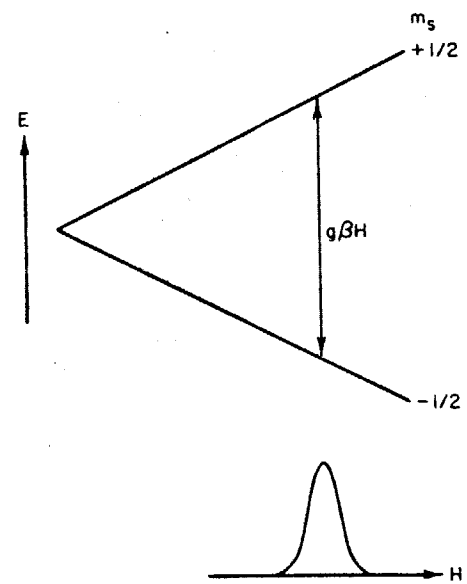

FIG. 6. Energy-level diagram for an $S = \frac{1}{2}$ state in an applied magnetic field H. The resultant absorption line is shown schematically.

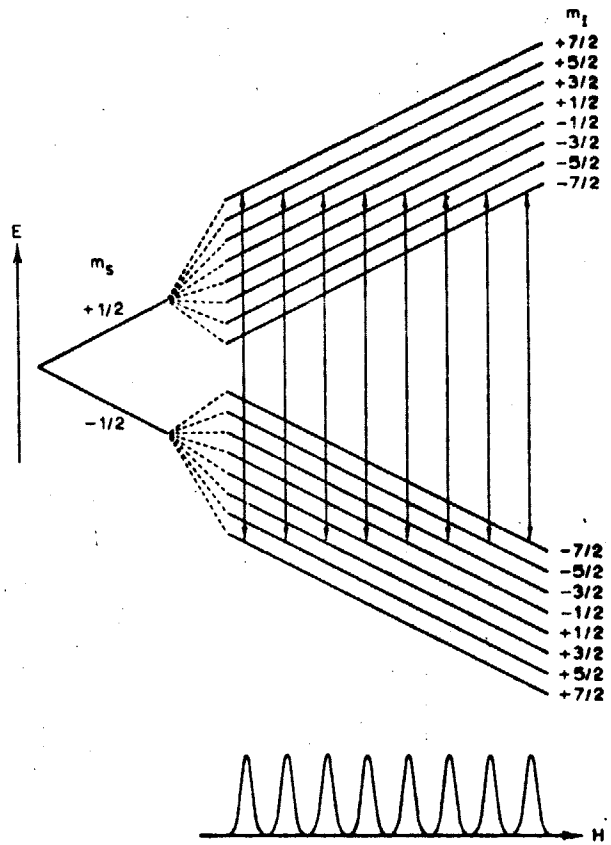

FIG. 7. The interaction of an $S = \frac{1}{2}$ state with a ^{59}Co nucleus ($m_I = 7/2$) in applied magnetic field H. The resultant hyperfine absorption lines are shown schematically.

whose nucleus has nonzero nuclear spin, then there is an interaction between the magnetic moments of the electronic and nuclear spin such that each electron spin-state is split into $2I + 1$ states, as shown in Fig. 7, for ^{59}Co($I = 7/2$). The resonance condition is now

$$h\nu = g\beta H m_s + \Sigma A m_s m_I \tag{10}$$

where A is a hyperfine interaction constant. The distance between two lines is $\Delta H = A/g\beta$. Normally only transitions in which $\Delta m_s = \pm 1$, and $m_I = 0$ are observed. If the electron interacts with additional magnetic nuclei there may be "superhyperfine" structure; for example, if there was

interaction with one nitrogen (^{14}N; $I = 1$) we would expect each hyperfine line to split into three. If the interaction was with two equivalent ^{14}N nuclei, we would expect fine lines with intensity ratio 1:2:3:2:1 because of the number of different ways of realizing the resultant total nuclear spin states (Fig. 8).

It is usual to describe spectra in terms of the two parameters g and A. The g-factor is a tensor, and its magnitude may therefore depend on the orientation of the molecule in the applied field. It may usually be represented by three values, g_{xx}, g_{yy}, and g_{zz}; x, y, and z being mutually perpendicular. A special case which is often found has $g_{xx} = g_{yy} = g_\perp \neq g_{zz} = g_\parallel$. Like g, A is also a tensor and may have values A_{xx}, A_{yy}, and A_{zz}.

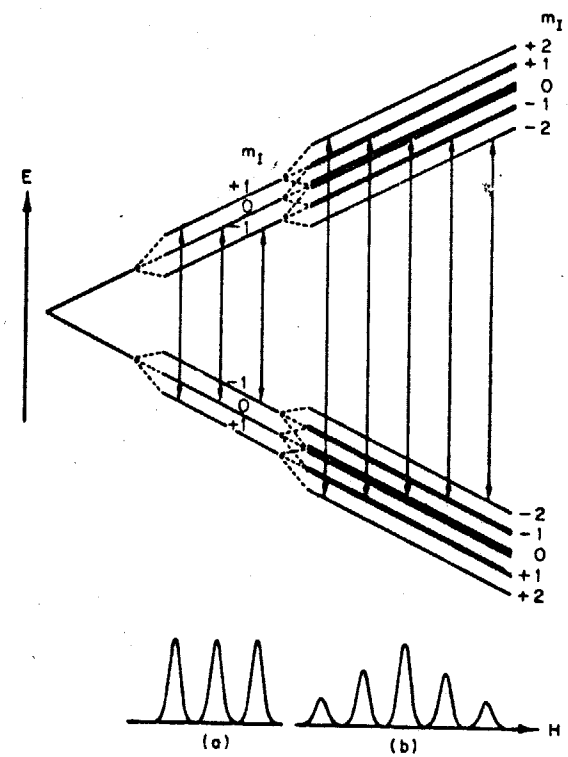

FIG. 8. The interaction of an $S = \frac{1}{2}$ with (a) one ^{14}N nucleus ($m_I = 1$) and (b) two ^{14}N nuclei ($m_I = 2$) in an applied magnetic field H. The resultant absorption lines are shown schematically.

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The spectrum of a magnetically dilute single crystal in which all the molecules, which have axial symmetry, are identical, gives rise to one signal with a g-factor given by Eq. (11)

$$g^2 = g_{\parallel}^2 \cos^2 \theta + g_{\perp}^2 \sin^2 \theta \tag{11}$$

together with associated hyperfine and superhyperfine structure if present.

In solution, where the molecule is tumbling rapidly and average g-factor is observed, e.g. Eq. (12).

$$g_{av} = \tfrac{2}{3} g_{\perp} + \tfrac{1}{3} g_{\parallel} \tag{12}$$

with associated average hyperfine structure.

These two cases are unlikely to be common in work with biological samples; the former because crystalline coenzymes are rare and, in any case, it is often of interest to follow the EPR as the reaction proceeds; the latter because it is unlikely that cobalt(II) derivatives, for reasons given below, will give spectra at temperatures where most systems are in the liquid state.

Therefore a more relevant state may be that of a frozen solution, polycrystalline material, or glass. Fortunately, it has been shown[14–16] that it is possible to derive much information from such spectra.

If one assumes that the molecules are oriented randomly with respect to the field, then it can be shown that discontinuities will occur in the absorption spectrum at certain orientations of the molecule to the field. For a molecule with axial symmetry, these discontinuities will occur when the angle between the tetragonal axis and the applied field is 0° and 90° corresponding to $g_{\parallel}$ and $g_{\perp}$ as shown[17] in Fig. 9 for a case in which $m_I = 0$. Since $g_{\perp}$ corresponds to both the x and y axes and $g_{\parallel}$ to the z axis only, there will be a statistical accumulation of signals around the position of $g_{\perp}$. When $g_x \neq g_y \neq g_z$ then all three g-values normally can be determined[17] as in Fig. 10.

The situation is[18–20] more complex when $m_I \neq 0$. For some transitions between the m_I levels, there may be additional discontinuities at a value of θ which is not immediately related to any important property of the complex. However, it is usually possible to measure $g_{\parallel}$ and $A_{\parallel}$ and any

[14] B. Bleaney, *Proc. Phys. Soc.* (*London*) **63A**, 407 (1950).
[15] R. H. Sands, *Phys. Rev.* **99**, 1222 (1955).
[16] J. D. Swalen and H. M. Gladney, *IBM J. Res. Develop.* **8**, 515 (1964).
[17] F. K. Kneubühl, *J. Chem. Phys.* **33**, 1074 (1960).
[18] R. Neiman and D. Kirelson, *J. Chem. Phys.* **35**, 156 (1961).
[19] H. R. Gersmann and J. D. Swalen, *J. Chem. Phys.* **36**, 3221 (1962).
[20] T. Vänngard and R. Aasa, *in* "Proc. Intern. Conf. Paramagnetic Resonance, 1st, Jerusalem 1962" (W. Low, ed.), Vol. 2, p. 509. Academic Press, New York, 1966.

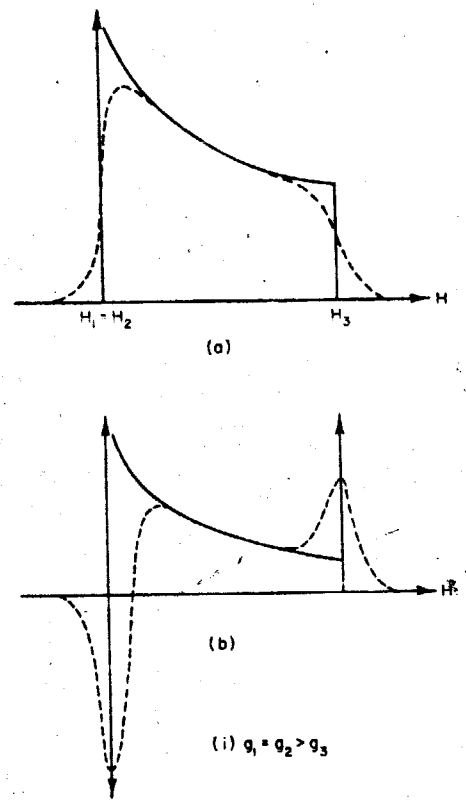

FIG. 9. Ideal (———) and line-broadened (- - - - -) (a) absorption and (b) derivative resonances from a polycrystalline sample which has $g_1 = g_2 > g_3$ or $g_x = g_y > g_z$ and $m_I = 0$. After Kneubühl.[17]

associated superhyperfine structure. It is much more difficult to obtain an accurate value of $g_{\perp}$ and $A_{\perp}$ since it is normally in the $g_{\perp}$ region that additional lines interfere.

Factors Affecting Line Widths

Relaxation Times. Similar considerations as those discussed for NMR serve to describe the interaction of the electron spin states with the environment. However, there are a number of important differences in the way the experimental conditions affect the line widths and, hence, resolution.

The principal mechanism by which the electron spin-states interact with the lattice is via its associated orbital motion. We saw that in many

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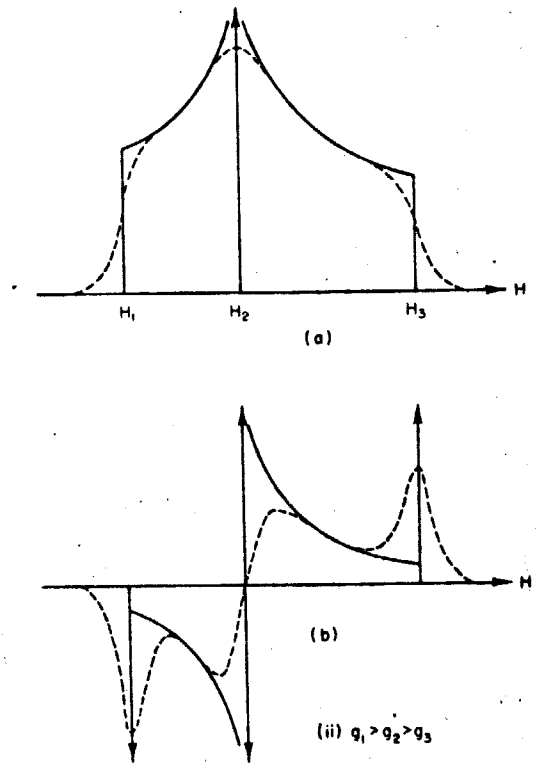

FIG. 10. Ideal (———) and line-broadened (- - - - -) (a) absorption and (b) derivative resonances from a polycrystalline sample which has $g_1 > g_2 > g_3$ or $g_x > g_y > g_z$ and ($m_I = 0$). After Kneubühl.[17]

molecules the angular momentum resulting orbital motion is "quenched," although some remains as a result of spin-orbital coupling. The latter is most important in determining line widths. In organic radicals, which usually consist of light atoms, spin-orbit coupling is small and the spin-lattice relaxation time correspondingly long. Consequently, the line widths are narrow even at room temperature. Concomitantly, the spectra are easily saturated so low power, high attenuation of the microwave power is usually necessary. With transition-metal complexes, the spin-orbit coupling is larger and the spin-lattice relaxation times shorter. Therefore, many paramagnetic transition-metal complexes do not even show EPR spectra at room temperature because T_{1e} is so short that line widths are large that the line widths are not observable. The presence of low-lying energy levels which have orbital contributions can provide an additional spin-lattice

relaxation mechanism, and many ions, e.g., high spin Co^{2+}, give EPR spectra only at very low temperatures. Low-spin Co^{2+}, d^7 complexes give spectra at 77°K but the latter broaden as the temperature increases.

Spin-spin relaxation imposes an additional limitation on possible experimental conditions. Increasing the concentration of the paramagnetic species normally leads to broadening by the dipolar interaction between the electron spins which is described by T_{2e}. One must normally use concentrations not greater than 10^{-2} M. However, because even *solid* B_{12r} can be considered magnetically dilute, it has been possible[21] to observe the EPR spectrum of solid B_{12r} although the broadening obscured some of the superhyperfine structure.

One advantage of broad lines in the EPR spectra of transition-metal complexes is that it is less easy to saturate the signal. Consequently, it is possible to work at high power, low attenuation. Since organic radicals are easily saturated, one must be careful to work over a range of attenuations to observe both organic and inorganic resonances. The difference between the saturation properties provides a method of distinguishing signals.

Modulation. For technical reasons it is possible to improve considerably the S:N ratio by modulating the applied field with an oscillating magnetic field. Within certain limits it is preferable to use an oscillating field of high frequency, 100 kHz is one commonly in use. The higher the amplitude of the modulating field, the greater the S:N ratio, but this leads to distorted signals and loss in resolution. Consequently the modulation amplitude should normally not be greater than the line width. If sensitivity is not a problem, but resolution is, then it is preferable to use a modulation amplitude one-tenth that of the line width.

The Properties of Cobalt(II)

The magnetic properties of a cobalt(II) ion depend on the ligands and the symmetry of the complex. When a free ion is introduced into a complex, the five d-orbitals no longer have the same energy because of their different symmetry properties (Fig. 11). In an octahedral molecule, the $d_{x^2-y^2}$ and d_{z^2} have the same symmetry properties as do the d_{xy}, d_{xz}, and d_{yz} orbitals (Fig. 12a). The former coincide with the axes, and take part in σ-bonding with the ligand whereas the latter can only take part in π-bonding which is weaker. The energy of the ligand electrons is lowered by interaction with the metal and those of the metal $d_{x^2-y^2}$ and d_{z^2} raised by bonding though, of course, it is an approximation to refer to them as "ligand" or "metal" electrons since there will always be some degree of covalency.

[21] R. Yamada, S. Schimizu, and S. Fukui, *Arch. Biochem. Biophys.* **117**, 675 (1966).

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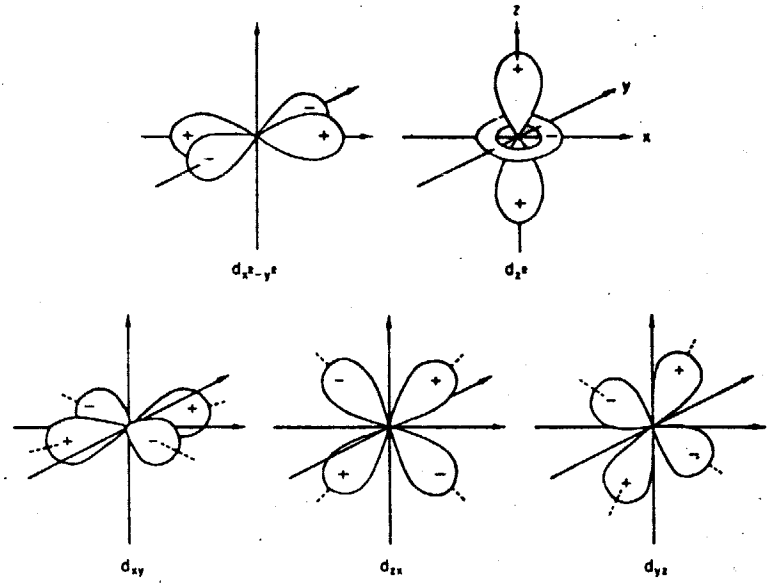

FIG. 11. Schematic representation of the 3*d*-orbitals (axes as for d_{z^2}).

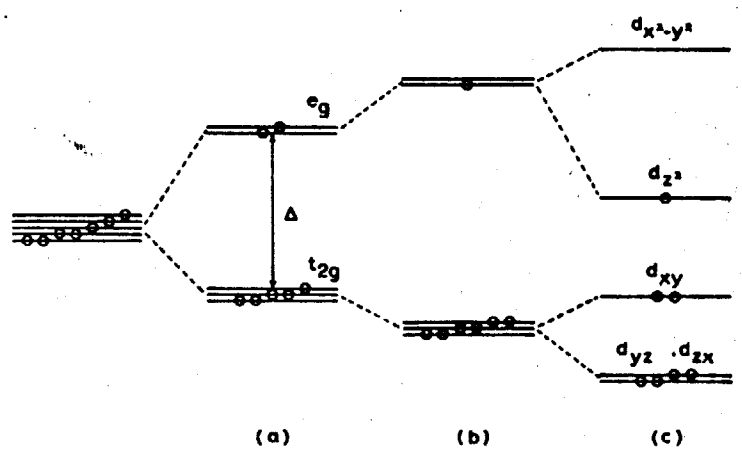

FIG. 12. The splitting of the *d*-orbitals by interaction with six ligand atoms; (a) octahedral high-spin complex, (b) octahedral low-spin complex, and (c) tetragonally distorted octahedral complex.

There remain two possible electronic configurations: $(t_{2g})^5$ $(e_g)^2$ (Fig. 12a) or $(t_{2g})^6$ $(e_g)^1$ (Fig. 12b). The former, high-spin, case occurs when the energy required to place electrons in the same orbitals (the pairing energy P) is greater than the separation between t_{2g} and $e_g(\Delta)$. The latter, low-spin, case has $\Delta > P$. Where the symmetry is lowered, say by having the two axial ligands different from the planar ligands, the $d_{x^2-y^2}$ and d_{z^2} no longer have the same symmetry and the orbital energy level diagram is that shown in Fig. 12c, which results if the in-plane bonding is stronger than the bonding to the axial ligands.

What are the magnetic properties of this electronic configuration with one unpaired electron? Because the state is not orbitally degenerate, we expect that the orbital contribution will be quenched and the magnetic moment close to the spin-only value, 1.73 B.M., and consequently an average g-value not far from that of the free electron. We expect it to be anisotropic with $g_z \neq g_x$ and g_y. Since the electron is well described by d_{z^2}, we do not expect it to have an orbital angular momentum about the z-direction; therefore $g_{\parallel} \simeq 2$. The other two g-values will be greater than 2 because of spin-orbit coupling. Associated with both g-values we expect to observe hyperfine coupling with the cobalt and axial ligands coordinated via atoms having magnetic nuclei.

Preparation of Vitamin B_{12r}

Cobalt(II) derivatives of cobalamins and cobinamides can be prepared by a number of methods: photolysis of cobalt-alkyl derivatives, reduction by zinc and hydrochloric acid, stannous chloride in acid, thiols, ascorbate, hydrogenation by hydrogen using a platinum catalyst and controlled potential electrolysis (CPR). The interpretation of spectra of cobalt(II) derivatives produced by the last two methods is more straightforward since neither the reductants nor their products can coordinate to the cobalt. Photolysis, reduction by thiols, Pt/H_2, or CPR can be used in both aqueous and nonaqueous solution. We have found that resolution is improved by the presence in solution of organic molecules, e.g., ascorbate or thiols, or the use of an organic solvent. Our preferred medium when using Pt/H_2 or CPR is propane-1,2-diol–water, 1:3.

Results

The initial investigations[22,23] of the EPR of B_{12} derivatives by Hogenkamp and co-workers, showed that the coenzyme, which did not give an

[22] H. P. C. Hogenkamp, H. A. Barker, and H. S. Mason, *Arch. Biochem. Biophys.* **100**, 353 (1963).
[23] H. P. C. Hogenkamp, J. E. Rush, and C. S. Swenson, *J. Biol. Chem.* **240**, 3641 (1965).

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EPR spectrum, photolyzed to give a spectrum identical to that of authentic B_{12r}. Photolysis of a frozen solution of DBC coenzyme gave rise to a spectrum containing both the cobalt(II) signal and that of an organic radical, presumably that of the adenosyl moiety which disappeared on melting and refreezing. Although hyperfine splitting $A_{||}$ was observed, no superhyperfine splitting could be resolved. A preliminary report showed[24] that the acidic (base-off) and basic (base-on) forms of vitamin B_{12r} have markedly different spectra. This was confirmed and extended in later publications[25,26] from two laboratories. The results on the base-on form were in accord. The spectrum of the base-on cobalamin B_{12r} (Fig. 13), which is

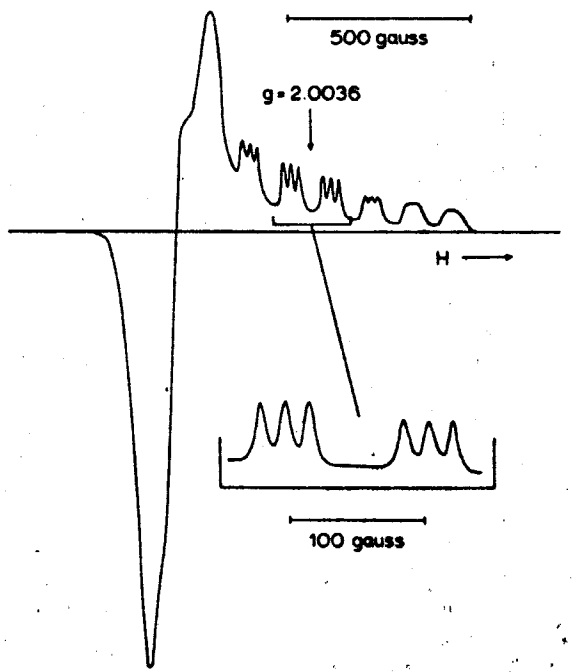

FIG. 13. The EPR spectrum of base-on cobalamin, B_{12r}, prepared by Pt/H_2 reduction in propane-1,2-diol–water, 1:4, at pH 7 measured at 100°K using X-band irradiation. (The enlargement shows the superhyperfine structure due to interaction with one ^{14}N nucleus of 5,6-dimethylbenzimidazole.)

[24] H. A. O. Hill, J. M. Pratt, and R. J. P. Williams, *Proc. Roy. Soc.* (*London*) **288A**, 352 (1965).
[25] G. N. Schrauzer and L.-P. Lee, *J. Am. Chem. Soc.* **90**, 6541 (1968).
[26] S. A. Cockle, H. A. O. Hill, J. M. Pratt, and R. J. P. Williams, *Biochim. Biophys. Acta* **177**, 686 (1969).

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similar to that of earlier work, has very well-resolved superhyperfine structure on the cobalt hyperfine peaks due to coupling with the nitrogen of the coordinated 5,6-dimethylbenzimidazole. The spectrum is typical of a polycrystalline sample of a molecule with axial symmetry, $g_\perp = 2.32$, $g_{||} = 2.004$ with the cobalt hyperfine well resolved for $g_{||}$, $A_{||\,Co} = 100 \times 10^{-4}$ cm^{-1} and $A_N = 17.3 \times 10^{-4}$ cm^{-1}. The spectrum is slightly sensitive to reductant and medium as shown in Table IV suggesting some interaction with the cobalt. The hyperfine and superfine splitting-parameters are sensitive to the coordinated nucleotide. Considering only nucleotides as sixth ligands (Table IV), there is an inverse correlation between $A_{||\,Co}$ and A_N which may be related to the changing length or strength of the Co-N bond. The spectra are consistent with an electronic configuration in which the unpaired electron occupies a d_{z^2} orbital giving $g_{||} \simeq 2$, $g_\perp > 2$.

There is some disagreement over the spectrum of acidified B_{12r} which, like all cobalamins, takes part[27] in a pH-dependent equilibrium which involves the removal of the 5,6-dimethylbenzimidazole from the coordination sphere of the cobalt, its concomitant protonation and presumed replacement by water with a $pK \sim 2.5$. The EPR spectra of the base-on and base-off forms are[26] very different. The spectrum of the base-off form (Fig. 14) is similar to that of factor B_r, Co(II) cobinamide. The spectrum of the base-off form reported[25] by Schrauzer seems to be that of the base-on form at lower resolution.

An EPR investigation[26] of the coordination of pyridine to the base-on form shows that even in 90% pyridine-water the formation of the pyridine species is not complete. However, for the pyridine species $A_{||\,Co} = 102 \times 10^{-4}$ cm^{-1} and further splitting is observed due to *two* coordinated nitrogen ligands with $A_N = 13.4 \times 10^{-4}$ cm^{-1}. Likewise factor B_r has a low-formation constant for the *second* pyridine. It was suggested[25] that the reluctance to coordinate pyridine showed that B_{12r} is five-coordinate under the conditions of the EPR experiment. Though it is possible that B_{12r} is five-coordinate in solution at room temperature, we do not consider that these EPR experiments differentiate between five-coordination and six-coordination with a solvent molecule as sixth ligand.

The Application of EPR to the Study of B_{12}-Dependent Enzymes

If it is possible to prepare a solution of an enzyme which is 10^{-3} to 10^{-4} M in B_{12} it should be possible to detect nitrogen superhyperfine structure in an EPR spectrum measured at −180°C. If only more dilute samples are available it will still be possible to detect the strong signal with $g \simeq 2.3$.

[27] R. A. Firth, H. A. O. Hill, B. E. Mann, J. M. Pratt, and R. G. Thorp, *Chem. Commun.* p. 1013, 1967.

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TABLE IV

EPR OF B_{12} DERIVATIVES MEASURED AT X-BAND AT 100°K USING FROZEN SOLUTIONS

B_{12} derivative[a]	Reductant	$g_1 \pm 0.001$	$g_1 \pm 0.01$	$A_{1\,Co} \pm 1$ $\times 10^4$ cm^{-1}	$A_N \pm 0.2$ $\times 10^4$ cm^{-1}
Co(II) cobalamin B_{12r} (base-on), pH 7	CPR[b]	2.004	2.32	100	17.3
	Pt/H_2[c]	2.004	2.32	100	17.3
	Ascorbate[d]	2.003	2.33	104	17.3
	Cysteine[d]	2.004	2.33	103	17.1
	Glutathione[d]	2.004	2.33	103	17.1
Co(II) cobalamin B_{12r} (base-off), pH 0	CPR[b]	2.006	$g_1 = 2.60$, $g_2 = 2.2 \pm 0.1$	135	—
	Pt/H_2[c]	2.006	$g_1 = 2.60$, $g_2 = 2.2 \pm 0.1$	135	—
	Ascorbate[d]	2.008	$g_1 = 2.60$, $g_2 = 2.2 \pm 0.1$	141	—
	Cysteine[d]	2.005	$g_1 = 2.50$, $g_2 = 2.2 \pm 0.1$	129	—
	Glutathione[d]	2.005	$g_1 = 2.46$, $g_2 = 2.2 \pm 0.1$	128	—
Co(II) cobinamide factor B_7, pH 3	Pt/H_2[c]	2.005	$g_1 = 2.57$, $g_2 = 2.2 \pm 0.1$	134	—
Factor B_7 + pyridine (1:1 complex), pH 7	Pt/H_2[c]	2.004	2.32	103	17.5
5-Hydroxybenzimidazole B_{12r}	Pt/H_2[c]	2.004	2.32	101	17.1
5-Methoxybenzimidazole B_{12r}	Pt/H_2[c]	2.004	2.33	101	17.1
2-Thiomethyladenine B_{12r}	Pt/H_2[c]	2.004	2.34	105	16.9
2-Methyladenine B_{12r}	Pt/H_2[c]	2.004	2.35	105	16.7
Adenine B_{12r}	Pt/H_2[c]	2.004	2.34	106	16.5

[a] Concentration ~10^{-3} M.
[b] Controlled potential reduction in propane-1,2-diol–water, 1:4, at mercury cathode at −0.5 V V° SCE (saturated calomel electrode).
[c] Catalytic reduction of the cyanocobalt(III) derivative in propane-1,2-diol–water, 1:4.
[d] Aqueous solution of the reductant.

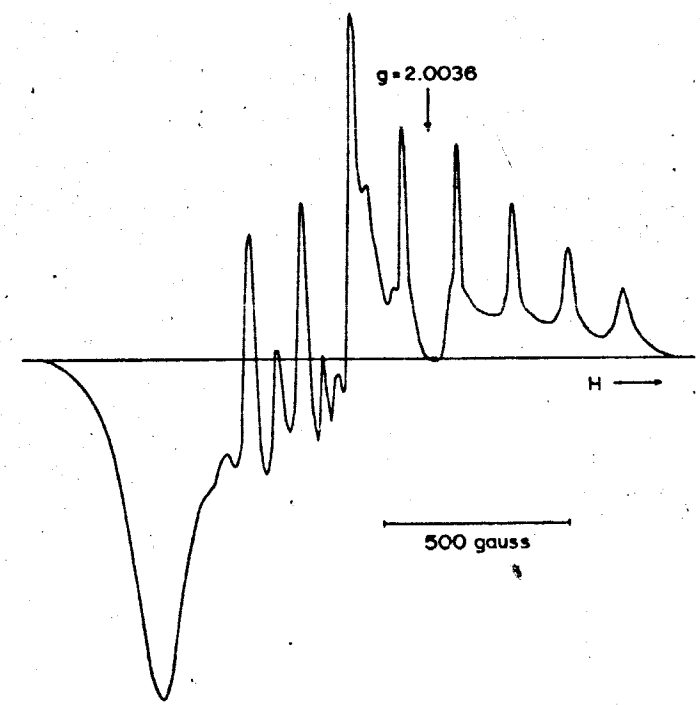

FIG. 14. The EPR spectrum of base-off cobalamin, B_{12r}, at pH 0 in propane-1,2-diol–water, 1:4, measured at 100°K using X-band irradiation.

For structural studies, therefore, more concentrated solutions are desirable but kinetic experiments using the time-dependence of the height of the $g = 2.3$ signal will be possible with dilute solutions. Free radicals formed from the coenzymes or substrates during the enzymatic reaction could be detected at much lower concentrations, provided their natural linewidths were not increased by the proximity of a cobalt(II) cobalamin. Extensive interaction would lead to an EPR spectrum which was not the sum of those of the individual components.

Acknowledgments

We are grateful to M. R. C. for financial support, Glaxo Research Ltd. and Professor K. Bernhauer for gifts of B_{12} derivatives. We thank Dr. J. Beaconsall for 220 MHz spectra, Dr. B. E. Mann for discussions, and Mr. S. A. Cockle for his careful work and help with the preparation of the manuscript.

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SOME OBSERVATIONS ON VITAMIN B_{12} BINDING PROTEINS

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Covalent coupling of vitamin B_{12} to high molecular weight carriers

Aquocobalamin was coupled covalently to poly-L-lysine or albumin by use of a carbodiimide. In a typical experiment 100 mg (73 μmoles) of aquocobalamin was mixed with 160 mg of 1-ethyl-(3-dimethylaminopropyl)-carbodiimide and 10 mg (78 μmoles lysine residues) of poly-L-lysine in 2 ml of 0·05 M-tris, pH 8·2. The sample was rotated at 37°C for 18 hours and then subjected to gel filtration on Sephadex G-50 in 0·05 M-tris, pH 7·2, with 1 M-NaCl. From measurements of the optical density at 355 nm of the two peaks eluted, the uptake of vitamin B_{12} on poly-L-lysine was 20 per cent, corresponding to a mean of 56 B_{12} molecules per poly-L-lysine molecule, or 2 mg B_{12} per mg poly-L-lysine. Coupling of aquocobalamin to albumin was carried out using a similar protocol. Cerous hydroxide-splitting of the covalently bound vitamin B_{12} complex showed the site of coupling to be the phosphate group in the vitamin B_{12} (Fig. 1, position IV).

Coupling of 5′-deoxyadenosylcobalamin to poly-L-glutamic acid or succinylated γ-G globulin was performed essentially as for aquocobalamin, although in the dark. The uptake was 3 and 25 per cent respectively. On exposure to light 70 and 28 per cent of the vitamin was eluted as low molecular weight material on gel filtration, indicating the fraction that had been attached to the high molecular weight carrier through the deoxyadenosine moiety (Fig. 1, position I).

Demonstration of the steric orientation of vitamin B_{12} on binding

The steric orientation of vitamin B_{12} on binding to human sputum binder (SB), intrinsic factor (IF), transcobalamin I (TC I), and transcobalamin II (TC II) has been studied by use of these covalent coupled vitamin B_{12} preparations, and some cobalamin analogues kindly supplied by Dr L. Mervyn (Glaxo Laboratories Ltd).

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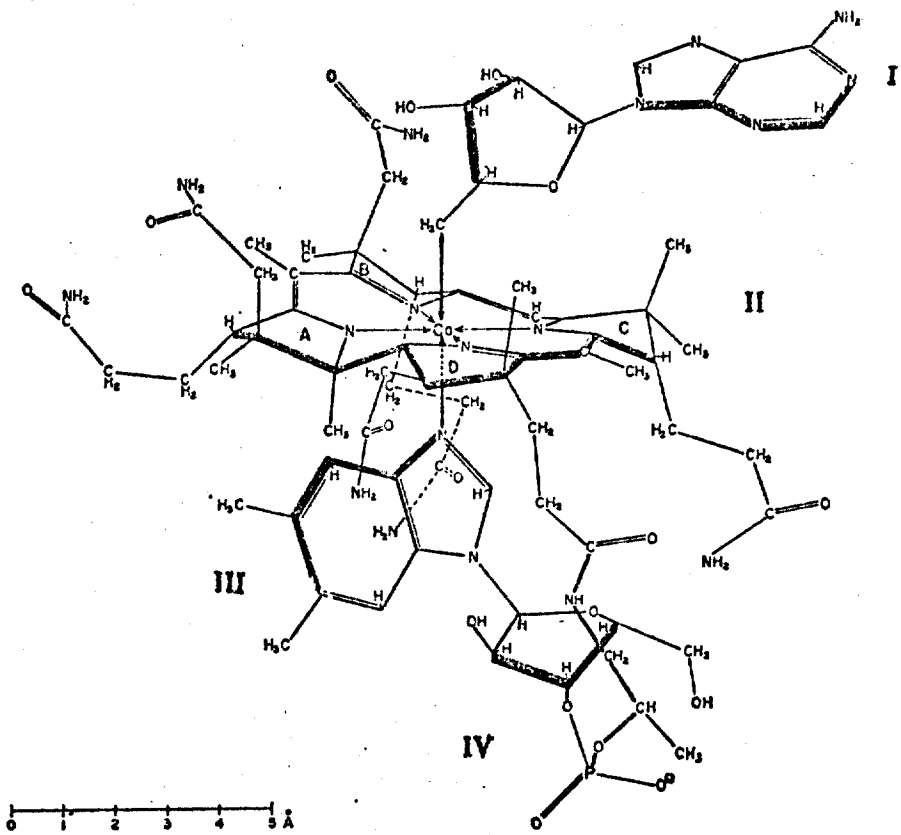


FIG. 1. Atomic positions in 5′-deoxyadenosylcobalamin. Adapted from Hodgkin (1964).

The ability to combine with the binding proteins was judged from the reduction in total binding capacity. A threefold excess of the B_{12} derivative was added to a known amount of B_{12} binder. After mixing at 4°C for 1 hour, a threefold excess of ^{57}Co–B_{12} was added, and the free and bound vitamin was separated by Sephadex G-25 gel filtration. The difference between total binding capacity and the amount of ^{57}Co–B_{12} bound was assumed to be occupied by the B_{12} analogue investigated. In order to facilitate comparison, the binding of the B_{12} analogue was expressed as a percentage of the binding capacity for aquocobalamin.

From the results summarized in Table I, it is concluded that the area of contact for the four binders is in the region of the edge of the B ring in the corrin nucleus, probably with overlap to the adjoining part of the dimethylbenzimidazole moiety.

TABLE 1

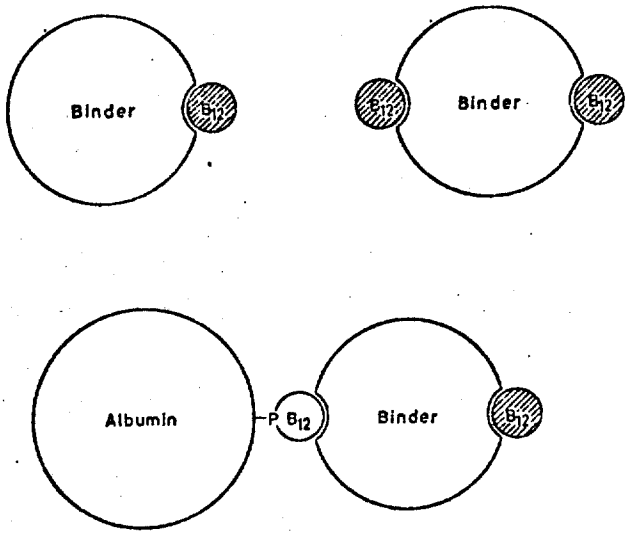
ANALOGUE COMPETITION FOR VITAMIN B_{12} BINDING SITES ON HUMAN B_{12}-BINDING PROTEINS. AMOUNT OF B_{12} DERIVATIVE BOUND AS A PERCENTAGE OF THE BINDING CAPACITY FOR AQUOCOBALAMIN

(SB = sputum binder, IF = intrinsic factor)

Binding moiety (see Fig. 1)	Cobalamin derivatives	SB	IF	TC I	TC II
I	Aquocobalamin	100	100	100	100
	5′-deoxyadenosylcobalamin	100	126	101	100
	IgG-5′-deoxyadenosylcobalamin	102	105	99	99
	Poly-D-glutamic acid-5′-deoxyadenosylcobalamin	102	37	96	58
II	Lactone-B_{12} (B ring)	97	13	81	91
	Anilide-B_{12} (C ring)	100	125	97	98
	Cobinamide	100	64	93	87
III	Pseudo-B_{12}	102	0	80	94
	Free dimethylbenzimidazole	5	0	16	43
IV	Albumin-B_{12}	100	103	100	86
	Poly-L-lysine-B_{12}	99	79	100	91

The number of combining sites on human vitamin B_{12}-binding proteins

The method used for demonstration of the number of combining sites on a binding protein is shown in Fig. 2. The assumption was made that the

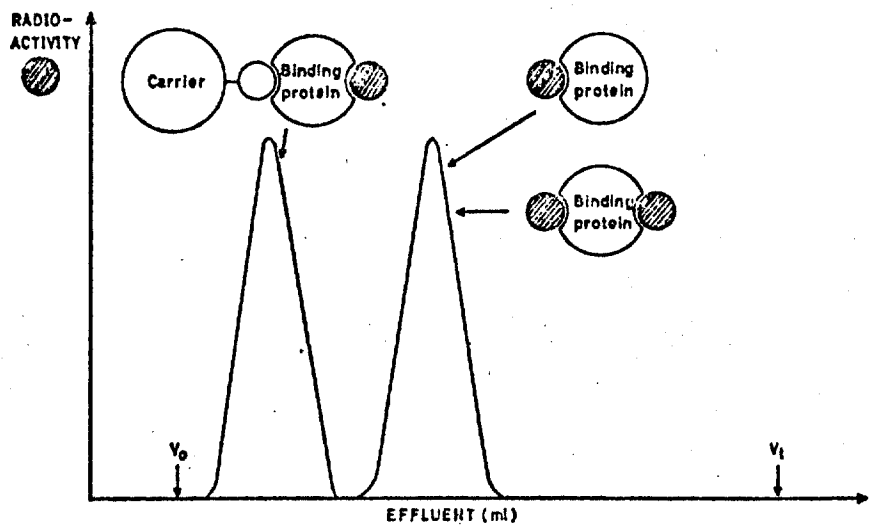


MODEL FOR DETERMINATION OF NUMBER OF COMBINING SITES ON A VITAMIN B_{12} BINDING PROTEIN

FIG. 2. Model for determination of number of combining sites on a vitamin B_{12}-binding protein.

occurrence of two or more binding sites on the protein would allow binding to the molecule of both $^{57}Co-B_{12}$ and albumin-coupled vitamin B_{12}. Such a complex, consisting of one molecule $^{57}Co-B_{12}$, one binding molecule, and one molecule of B_{12}-albumin, would have a bigger molecular size (Stokes radius) than a binder with only $^{57}Co-B_{12}$. Determination of Stokes radius was made by gel filtration on Sephadex G-200 as illustrated in Fig. 3.

An amount of the B_{12}-binding protein having a known binding capacity was mixed at 4°C with one-tenth to one-hundredth the binding capacity of $^{57}Co-B_{12}$. After 30 minutes an excess of unlabelled B_{12}-albumin was added and the mixture was subjected to gel filtration on Sephadex G-200 for determination of Stokes radius for the bound $^{57}Co-B_{12}$.



MODEL FOR DETERMINATION OF NUMBER OF COMBINING SITES BY SEPHADEX GEL FILTRATION

FIG. 3. Model for determination of number of combining sites by Sephadex gel filtration.

In order to confirm the suitability of the method, the number of combining sites on the vitamin B_{12}-binding protein in a homogenate of *Lactobacillus leichmannii* was also studied (Fig. 4). The increase in Stokes radius (Table 2) corresponds to an increase in molecular volume of about the volume of B_{12}-albumin. From this the presence of two combining sites is inferred.

Measurements of Stokes radius of the four human binding proteins (Table 2) trace-labelled with $^{57}Co-B_{12}$ and mixed with B_{12}-albumin, reveals that each of the binders has only one combining site.

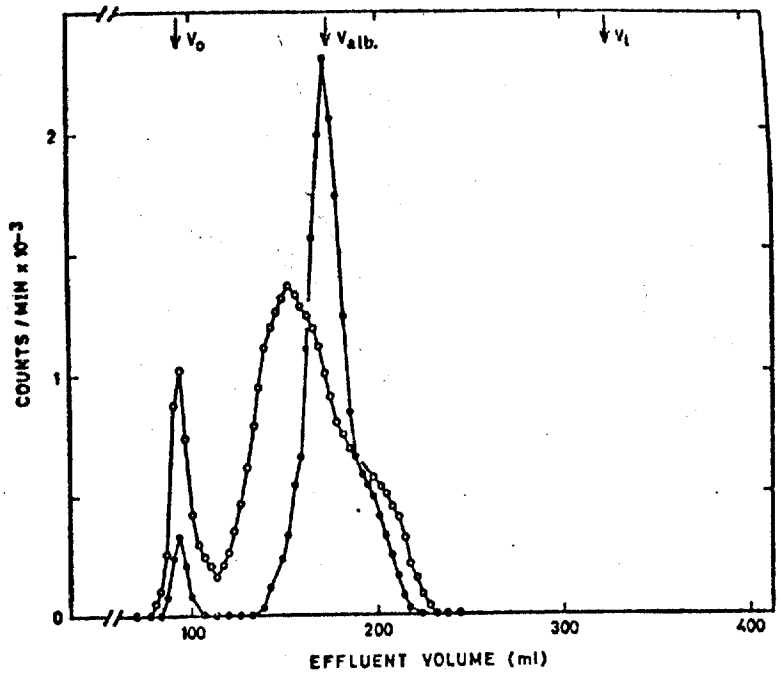


FIG. 4. Diagram of Sephadex G-200 gel filtration of vitamin B_{12}-binding protein from *Lactobacillus leichmannii* trace-labelled with ^{57}Co–vitamin B_{12} (●——●). The peak of ^{57}Co activity eluted at a position corresponding to a Stokes radius of 36·4 Å. On addition of B_{12}-albumin to the ^{57}Co–B_{12} trace-labelled binding protein (○——○) Stokes radius increases to 45·3 Å, indicating the presence of two B_{12}-binding sites.

TABLE 2

STOKES RADIUS OF VITAMIN B_{12}-BINDING PROTEINS

(Mixing time 30 minutes)

Vitamin B_{12}-binding protein	Binding capacity pmol	^{57}Co–B_{12} added pmol	Stokes radius (Å) of ^{57}Co–B_{12} and binder	
			B_{12}-albumin added (3 pmol)	No B_{12}-albumin added
Sputum binder	2·30	0·03	43·6	43·8
Intrinsic factor	2·65	0·03	33·5	32·8
Transcobalamin I	1·00	0·01	53·0	51·3
Transcobalamin II	3·00	0·03	24·8	25·5
L. leichmannii	0·40	0·03	45·3	36·4

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STRUCTURE OF VITAMIN B_{12}

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SINCE our communication in August 1955[1], we have carried our refinement of the structure of vitamin B_{12} a critical stage further. Four more calculations of the electron density distribution have been made, one for the wet B_{12} crystals, two for air-dried B_{12} crystals and one for the hexacarboxylic acid.

In Fig. 1 are shown the electron density peaks over all the atoms of the B_{12} molecule as it appears from our present calculations in the wet crystals. These peaks are rather lower than those observed for the hexacarboxylic acid owing to the smaller number of X-ray reflexions given by the larger molecule in proportion to its size. But they serve to show the relative positions of all the atoms in space of this very complex molecule within probably as little as 0·3 A. Similar evidence has been obtained for air-dried B_{12}; for both crystals the reliability indices in the latest structure factor calculations, 36·1 and 30·6 per cent, respectively, are now low

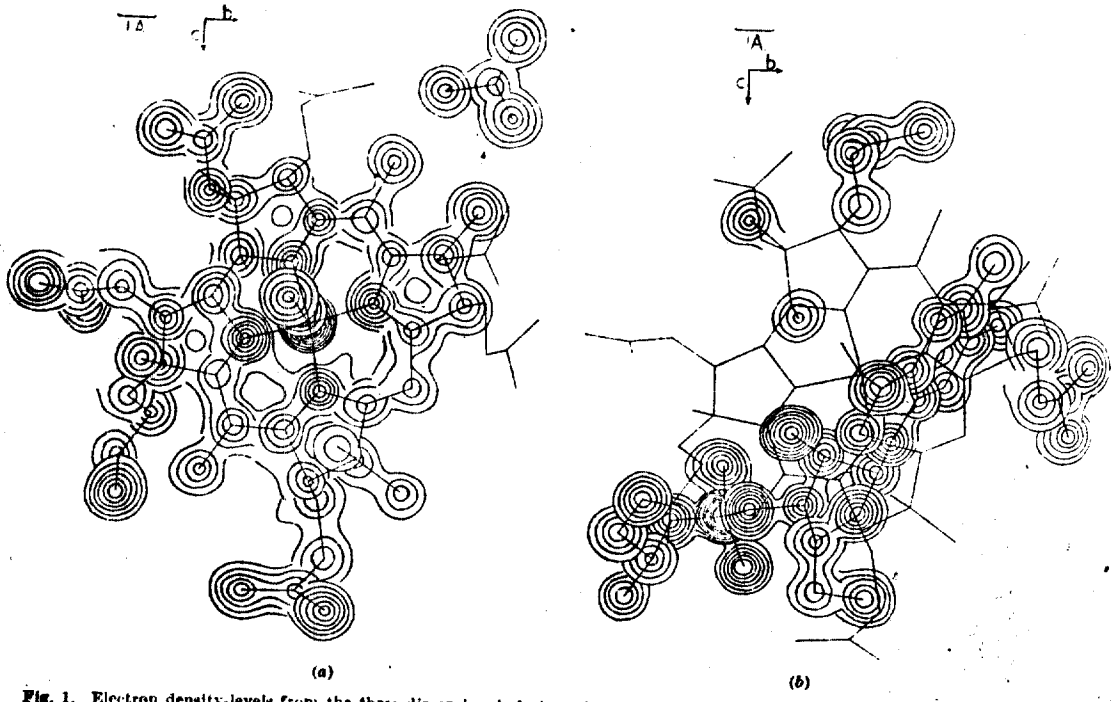


Fig. 1. Electron density-levels from the three-dimensional electron density distribution for wet B_{12} crystals, calculated with terms phased on 93 atoms

Here, and in Fig. 4, the contours are drawn in the sections of the calculated distribution parallel with the *a* plane, passing at or near the atomic positions. Owing to the complexity of the molecule, complete contours for every atom are not given and the figure is divided into two parts. (*a*) shows the cobalt-containing nucleus and cyanide group; (*b*) the benzimidazole, sugar, phosphate and propanolamine groups, the side chains being divided between the two. The acetamide chain on ring *B* is inset. The contour interval is 1 e/A.^{3}

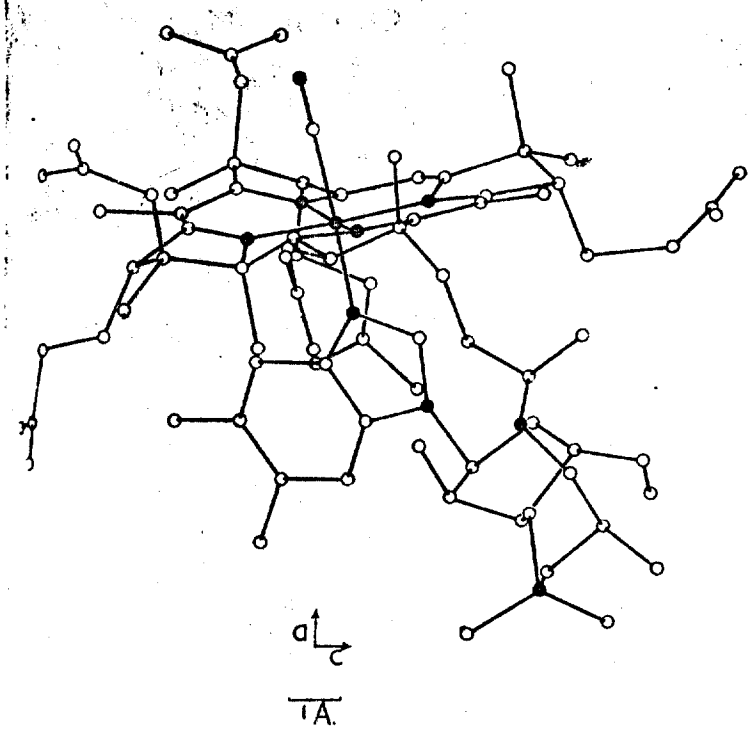
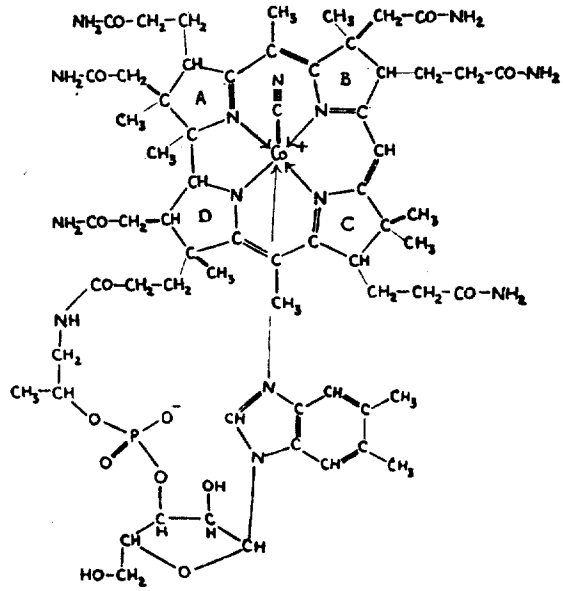


Fig. 2. The atomic positions found in the molecule of vitamin B_{12}. These are shown as derived in the wet crystals, projected on the *b* plane. To distinguish different parts of the molecule, bonds within the cobalt containing nucleus, benziminazole and cyanide groups are shown in black, together with the cobalt and phosphorus atoms and the nitrogen atoms of the nucleus, benziminazole, propanolamine and cyanide groups

enough to indicate that the solution we have reached is likely to be essentially correct.

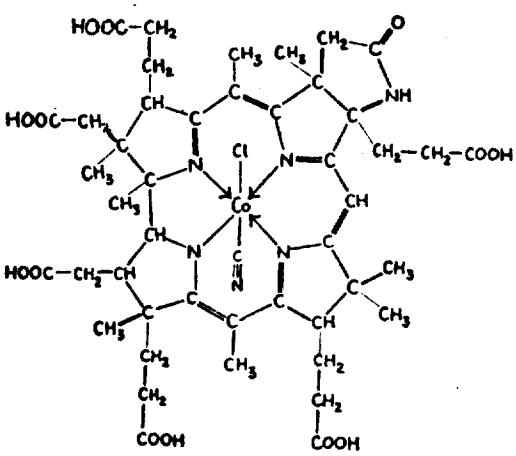
The molecule that appears is very beautifully composed, not far from spherical in form, with all the more chemically reactive groups on its surface. It is drawn in projection in Fig. 2. It is built around the two planes of the benziminazole nucleus and the central cobalt-containing nucleus, which are nearly at right angles to each other. The ribose ring turns in a position nearly normal to the benziminazole group, which permits its easy linking through the phosphate, propanolamine, and propionic acid residues to ring *D* of the planar group. The benziminazole nucleus is packed in on either side by the propionamide side-chains in the extended, staggered configuration, attached to rings *A* and *B*; the side-chain on ring *C*, in the gauche configuration, lies above the ribose ring. All the acetamide residues project from the opposite side of the nucleus to the propionamide residues, towards the cyanide group. It is an interesting point that one of them—that on ring *B*—swings round the carbon-carbon single bond from a position directed away from, to one in contact with, the cyanide group, when the crystals are removed from their mother liquor; this permits the rather closer packing of the molecules found in the air-dried crystals. Throughout the molecule, as shown by Figs. 1 and 2, the atomic positions found conform in a most convincing way with the stereochemical rules established by the study of simpler molecules.

It now seems reasonably certain that we should formulate vitamin B_{12}, $C_{63}H_{88}O_{14}N_{14}PCo$, as in I, and the hexacarboxylic acid as in II, with six double bonds in the inner ring of the central nucleus; these can form a resonating system by intervention of the cobalt atom as illustrated by III. Some evidence in support of this has been obtained by theoretical calculations[3], by spectroscopic measurements[3] and from further chemical studies[4]. Our own evidence for this formulation is the geometrical form of the nucleus, which is illustrated by Fig. 3, and which is closely similar in all three crystals studied. The interatomic distances shown in this figure are derived from the latest calculations on the hexacarboxylic acid and are not individually very precise; in other parts of the molecule distances differing by as much as 0·16 A. from accepted bond-lengths have been found. But it is notable that in the region N_6–N_{10} *all* the bond-lengths are shorter than normal single bond distances; their average value, 1·36 A., is similar to that found in the phthalocyanines[5]. The positions of C_{19} and C_{20}, one or other of which would be expected to be out of the plane of the central ring system if only five double bonds were present, are even more significant. In the hexacarboxylic acid they appeared at all stages of the refinement to be within 0·2 A. of this plane, but in the B_{12} crystals themselves their positions were at first more confused, particularly C_{20}. As a critical test, accordingly, this atom was omitted from the phasing calculations used for the latest electron distribution calculated for air-dried B_{12}. It has now appeared, as Fig. 4 shows, as a small but clearly defined peak at a site which is closely equivalent to that found in the hexacarboxylic



acid along the line Co to C_8. There is still some departure from planar character in this region of the inner nucleus in both wet and air-dried crystals, but this seems to affect N_{10}, C_9 and C_8 as much as C_{29}. If real, it may be a consequence of a small amount of compression from the benziminazole nucleus, which is in contact with these atoms. It is, in any event, not of the geometrical form that would be expected if C_8 were reduced.

The crystal structures of the different B_{12} compounds are still not completely solved; many of the water molecules which occupy space between the vitamin molecules have been placed, but not all. Still further small adjustments are to be expected on continued refinement of the atomic positions



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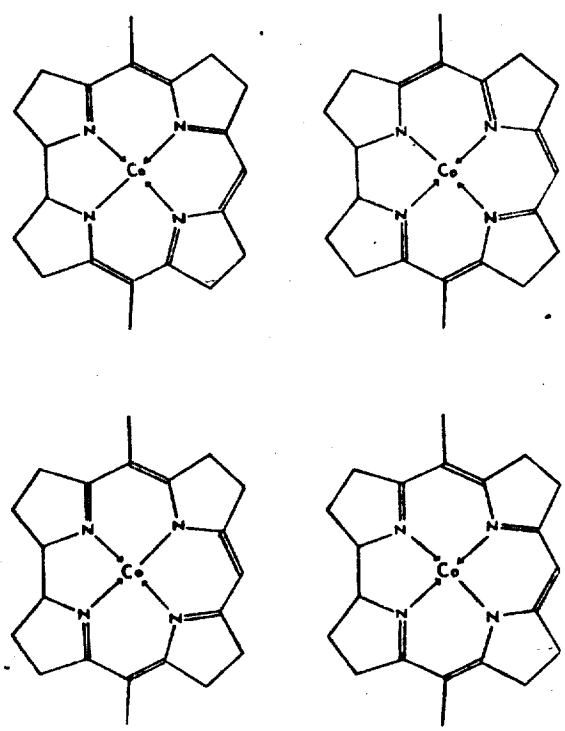

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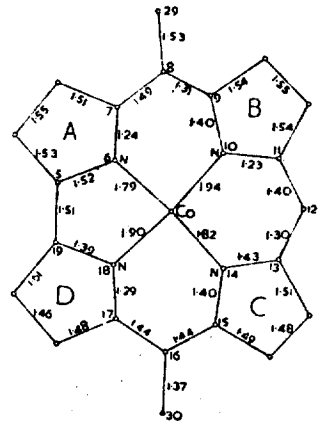

Fig. 3. Interatomic distances found in the inner nucleus of the hexacarboxylic acid, derived from nine refinements of the calculated electron density distribution

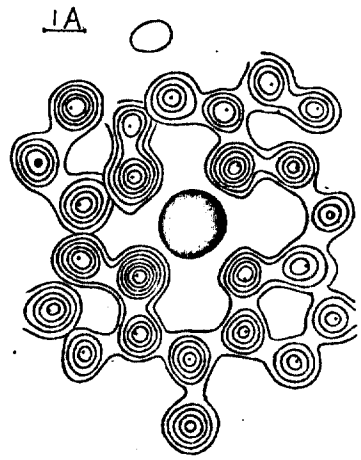

Fig. 4. Electron density peaks over the nucleus in air-dried vitamin B_{12}, derived from the three-dimensional electron density distribution calculated with terms phased on 95 atoms (Princeton data)

observed. We hope that future calculations will add still greater precision and certainty to our knowledge of the structure of the molecule.

The latest series of calculations on wet B_{12} were carried out on the University of Manchester electronic computer through the generous co-operation of Dr. D. W. J. Cruickshank, Miss Diana Pilling and Mr. J. F. P. Donavan, of the University of Leeds. Those on air-dried B_{12} and on the hexacarboxylic acid were made on the National Bureau of Standards automatic computer in Los Angeles, supported by the United States Office of Naval Research.

Again we acknowledge the very generous support given this research by the Nuffield Foundation, and materials and help given us by Dr. E. Lester Smith, Glaxo Laboratories, Ltd., and Dr. K. Folkers, Merck Laboratories. Two of us (J. P. and J. K.) received grants from the Department of Scientific and Industrial Research.

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Lab Invest. 13: 1394-1399

Investigations about the Site of Production of Castle's Gastric Intrinsic Factor

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Clinical studies performed on pernicious anemia patients in relapse by Fox and Castle[4] and by Landboe-Christensen and Plum[6] have shown that dried powdered human gastric fundus has intrinsic factor activity. This is in accordance with the postmortem finding of varying degrees of glandular atrophy in the fundus part of stomachs of patients who had suffered from pernicious anemia.[8] It is therefore generally accepted that in man the fundus glands are responsible for the elaboration of intrinsic factor.

In the rat the fundus is also believed to be the source of intrinsic factor. This was suggested by data obtained by Watson and Florey[12] and by our group[5, 9] about the effect of resection of various parts of the stomach on the absorption of labeled vitamin B_{12} in this species and on tests for intrinsic factor activity of extracts of these parts in rats which had been subjected to total gastrectomy.

Localization at a cellular level had been attempted by autoradiography of sections of rat stomach incubated with radioactive vitamin B_{12} with a high specific activity. This was based on the known affinity of intrinsic factor for vitamin B_{12}. In the rat selective uptake of the labeled vitamin B_{12} was observed in the chief cells, which are also considered to be responsible for pepsinogen production. In sections heated to 100° C. these cells did not take up the radioactive material. This is in agreement with the known thermolability of intrinsic factor.

In the present investigation we tried to confirm the specific nature of the binding of vitamin B_{12} in the chief cells by exposing sections of rat stomach to the γ-globulin fraction of serum of pernicious anemia patients with anti-intrinsic factor activity. In previous studies Abels *et al.*[1, 2] showed that some of these sera contain antibodies which inhibit specifically the B_{12} binding by intrinsic factor, but do not influence the binding capacity of some nonintrinsic factor substances with affinity for vitamin B_{12}.

We have also applied the autoradiographic procedure to human fundus mucosa both with and without previous exposure to the γ-globulin preparation with anti-intrinsic factor activity. In some experiments the anti-intrinsic factor γ-globulin was treated with lyophilized neutralized normal human gastric juice, a lyophilized extract of hog pylorus mucosa, or lyophilized hog fundus mucosa before it was applied to the sections of human stomach.

MATERIALS AND METHODS

In the studies on rat stomach we took this organ from adult male rats weighing 250 to 300 gm. immediately after they had been killed by decapitation.

The human stomach material was obtained from fresh surgical specimens from patients who had been subjected to a Billroth II partial gastrectomy for duodenal ulcer. If the fundus mucous membrane showed abnormalities, *e.g.*, gastritis on histologic examination it was considered unsuitable.

The radioactive vitamin B_{12} was labeled with Co^{57} or Co^{58}. Its specific activity at the time of delivery varied from 59.4 to 81.6 μc. per microgram for the Co^{58}-B_{12} and was 93.5 to 120.5 μc. per microgram for the Co^{57}-B_{12}.

The γ-globulin fractions used in the control experiments were obtained by chromatography on a diethylaminoethyl-

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cellulose column according to Levy and Sober[7] from a pool of normal sera and a pool of pernicious anemia sera. The patient sera had been shown to have a blocking effect on the B_{12}-binding power of neutralized normal human gastric juice as determined by Sephadex G-75 gel filtration.[1]

For the autoradiographic procedure we used 7-μ sections of fundus mucous membrane cut in a cryostat. These sections were airdried and fixed in saturated ammonium sulfate at 4° C. After a short rinse with phosphate buffer, pH 7.3 they were exposed to labeled vitamin B_{12} for 2 hours at room temperature. Subsequently, the slides were washed with three portions of the saturated ammonium sulfate. Then they were treated with gelatin-chromalum to prevent slipping of the film during the developing procedure and covered with the stripping film (Kodak autoradiographic plates AR-10). The material was developed after periods of exposure varying from 1 to 8 weeks. The sections were stained with a Giemsa solution in a buffer, pH 5.75.

The sections, which had been treated with the γ-globulin fractions, were incubated for 1 hour at 37° C., washed two times for periods of 10 minutes with the saturated ammonium sulfate and one short rinse in phosphate buffer, pH 7.3, to remove the ammonium sulfate crystals and exposed to the radioactive vitamin B_{12}.

RESULTS

The autoradiographs of the rat stomach gave a picture essentially similar to that previously described by Keuning, *et al.*[5] In the fundus glands very intensive blackening was confined to the basal zone (Fig. 1). Phase contrast microscopic study of the unstained sections as well as light microscopy of the Giemsa-stained sections (Fig. 2) showed that the chief cells had marked affinity for the radioactive material. The uptake of labeled vitamin B_{12} by these cells was abolished by pretreatment with the anti-intrinsic factor γ-globulin (Fig. 3, *A* and *B*). Treatment with normal γ-globulin had no effect on the distribution of vitamin B_{12}.

In the human stomach we observed a different picture. In this case it was the middle third of the glandular layer that showed blackening in small round spots

FIG. 1. Autoradiograph of unstained rat fundus with intensive blackening by Co^{57}-B_{12} over the basal part of the glands. Superficial zone is faintly visible at the *upper margin* of the photograph ×80.

FIG. 2. Autoradiograph of the same rat fundus as in Figure 1 with additional Giemsa staining to visualize the architecture of the mucosa. Radioactive blackening is confined to the chief cells. Parietal cell nuclei are seen in the white areas. ×350

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FIG. 3. Autoradiograph of the same unstained rat fundus as in Figure 1. Surface epithelium is faintly visible at the *upper margin*. *A*, Treated only with Co^{57}-B_{12}; *B*, exposed to anti-intrinsic factor γ-globulin fraction before treatment with Co^{57}-B_{12}. Blackening over the chief cells has been prevented. ×105.

(Fig. 4*A*). The anti-intrinsic factor containing γ-globulin inhibited the uptake of radioactive vitamin B_{12} by the middle part of the glandular layer (Fig. 4*B*). In agreement with the studies in the rat the normal γ-globulin fraction did not influence the uptake of the radioactive material. Pretreatment with anti-intrinsic factor γ-globulin previously absorbed with known intrinsic factor sources, including hog pyloric mucosa, did not cause inhibition of the uptake of vitamin B_{12}. However, hog fundus mucosa, which is known to have no intrinsic factor activity, did not block the anti-intrinsic factor antibody action in its preventing the uptake of radioactive vitamin B_{12} by the sections. The distribution and the shape of the cells which show blackening is highly characteristic for the parietal cells in the human stomach. This was confirmed by the study of the Giemsa-stained sections (Fig. 5).

DISCUSSION

The results obtained in the rat are in agreement with the data presented by Keuning, Arends, Mandema, and Nieweg,[5] who observed a marked affinity of the chief cells for vitamin B_{12}.

Treatment with the anti-intrinsic factor γ-globulin fraction abolished the uptake of the radioactive vitamin B_{12} by these cells. This anti-intrinsic factor antibody is known to inhibit the promotion of absorption and the binding of vitamin B_{12} by intrinsic factor of various species including the rat[1-3, 10, 11] and unpublished data. The effect of the anti-intrinsic factor γ-globulin fraction on the autoradiographs therefore provided significant support for the belief that the binding substance in the chief cells is indeed intrinsic factor. In agreement with this was also the lack of effect of pretreatment of the sections either with a normal γ-globulin fraction or anti-intrinsic factor

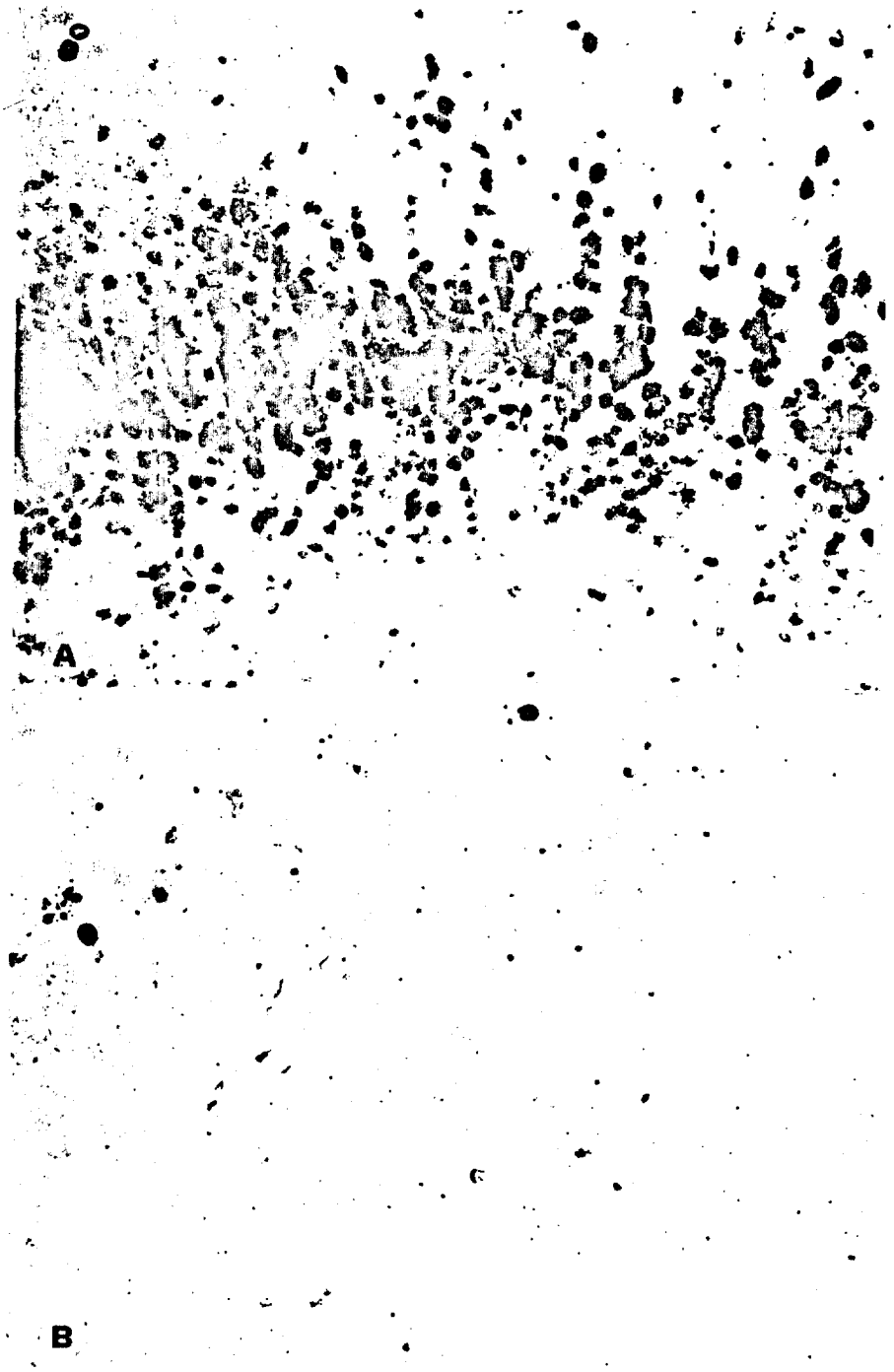

FIG. 4. Autoradiograph of human fundus after incubation with Co^{57}-B_{12}, unstained, showing the whole width of the glandular layer. *A*, Radioactive blackening is present in isolated spots, which increase in number from the basal part toward the middle zone, corresponding in size and outline with the parietal cells. ×80. *B*, Section exposed to anti-intrinsic factor γ-globulin fraction before treatment with Co^{57}-B_{12}. Radioactive blackening over parietal cells does not appear. ×80.

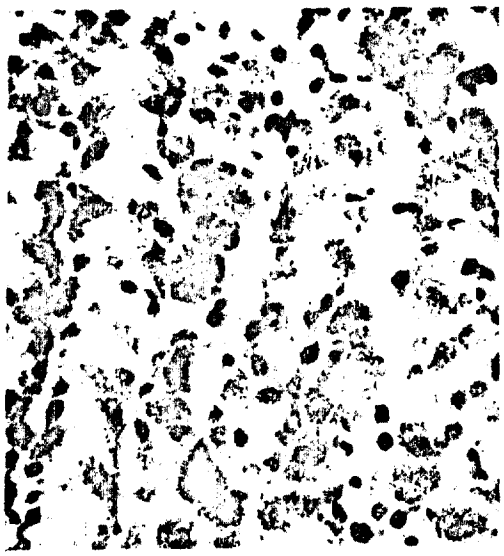

FIG. 5. Autoradiograph of human fundus treated with Co^{57}-B_{12} and stained with a Giemsa solution. The grains of radioactive blackening are clearly localized in the parietal cells lining the gastric tubules. ×500.

γ-globulin which had been mixed with a known source of intrinsic factor activity.

In the handling of the human material a more prolonged exposure was essential, for in previous studies using the same exposure time as in the original work on the rat, we did not obtain consistent results with human stomach. In the present investigation of the human gastric fundus, blackening was found in the glandular layer. Pretreatment with anti-intrinsic factor γ-globulin abolished the uptake in the glands. However, the specific affinity for vitamin B_{12} was observed not in the basal part of the glands as in the rat stomach, but mainly in the middle zone of the glandular layer. In the Giemsa-stained sections it appeared clearly that the blackening was confined to the cytoplasm of cells corresponding in size, outline, and distribution to parietal cells.

This result was rather surprising to us in view of the similarity in histologic appearance of the gastric fundus in the rat and in man. It seems that intrinsic factor is elaborated by different cells in the rat and in the human stomach. We have no explanation for the unexpected observation that a highly specialized cell such as the hydrochloric acid-secreting parietal cell may be involved in the production of a macromolecule with a highly specific carrier function.

Studies about the site of production of the intrinsic factor in other species are in progress.

SUMMARY

This paper reports some attempts to obtain information about the site of production of Castle's gastric intrinsic factor in the rat and in man by a combination of autoradiographic and immunologic procedures. In the rat previous studies by our group using autoradiography of sections of gastric fundus incubated with radioactive vitamin B_{12} had shown a strong affinity of the chief (pepsinogen) cells for the vitamin. The specificity of the vitamin B_{12} uptake by these cells was confirmed in the present investigations by pretreatment of the sections with anti-intrinsic factor γ-globulin. This anti-intrinsic factor γ-globulin fraction, which was derived from a pool of sera of pernicious anemia patients, had been shown to impair the binding of vitamin B_{12} by sources of intrinsic factor activity, but not by some nonintrinsic factor-binding substances.

The same procedure was applied to fresh human stomach specimens obtained at operation. In man, affinity for vitamin B_{12} was found in the parietal cells. The uptake of the labeled vitamin by these cells was inhibited by exposure to anti-intrinsic factor γ-globulin.

These data indicate that in man Castle's intrinsic factor is elaborated by the hydrochloric acid-secreting parietal cell, whereas in the rat the chief pepsinogen-producing cell is believed to be responsible for intrinsic factor production.

Acknowledgment. We wish to express our thanks to the N. V. Philips-Duphar, Amsterdam, for the gift of the radioactive vitamin B_{12}, which made this study possible.

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Scand. J. Haemat. (1968) 5, 64–74

The Vitamin B_{12} Binding Capacity of Transcobalamin I and II of Normal Human Serum

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The unsaturated serum vitamin B_{12} binding capacity ($UB_{12}BC$) of transcobalamin O, I and II (TC O, I and II) of 50 normal subjects was determined by gel filtration on Sephadex G-200. TC O bound from 0 to 76 pg. per ml. of serum, accounting for up to 5.2 per cent of the $UB_{12}BC$, while TC I bound from 72 to 1057 pg. per ml., or up to 47.1 per cent of the $UB_{12}BC$. TC II bound from 650 to 1891 pg. per ml., accounting for up to 90.3 per cent of the $UB_{12}BC$.

There was a significant positive correlation between the binding capacity of TC I and of TC II when each was compared with the total unsaturated B_{12} binding capacity. A positive correlation therefore existed between the binding capacity of TC I and that of TC II.

TC O, I and II were the only binders of added B_{12} over a range from 75 to 20,760 pg. per ml. of serum. The relative amount of B_{12} bound to TC I and to TC II remained fairly constant in an individual serum regardless of the amount of B_{12} added.

The vitamin B_{12} (B_{12}) in normal human serum is bound to an alpha globulin (Pitney et al. 1954). This protein has been called transcobalamin I (TC I) by Hall & Finkler (1964) and the alpha globulin B_{12} binder by Retief et al. (1967). Recent administration of B_{12}, either orally or parenterally, or *in vitro* addition to serum or plasma results in the greater part of the B_{12} being bound to a second binding protein, transcobalamin II (TC II) of Hall & Finkler (1964) or the beta globulin B_{12} binder of Retief et al. (1967). The endogenous B_{12} of normal human serum is all bound to TC I, none being detected bound to TC II by available fractionation methods (Mendelsohn et al. 1958, Gabuzda et al. 1965).

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Previous investigations on the individual TC I and TC II binding capacity have yielded somewhat differing results (Hall & Finkler 1966, Retief et al. 1967, Mikkonen et al. 1967). The present investigation was undertaken to determine the TC I and TC II binding capacities of serum from a group of normal subjects by gel filtration on Sephadex G-200. Five sera were further studied after addition of different amounts of B_{12} in order to determine whether it was necessary to saturate TC I before TC II would bind added B_{12} and to test the hypothesis that at high levels, other proteins are capable of binding B_{12}.

MATERIAL AND METHODS

Normal subjects

Serum was obtained from 50 hospital personnel, 39 females and 11 males, ranging in age from 18 to 42 years. The haemoglobin values and erythrocyte sedimentation rates were within the normal range. The specimens were frozen after collection and stored at −20° C. until used.

Serum vitamin B_{12} and unsaturated vitamin B_{12} binding capacity ($UB_{12}BC$) were estimated by the radioisotope dilution and charcoal adsorption method as described by Lau et al. (1965) and Gottlieb et al. (1965). The vitamin B_{12} content varied from 175 to 656 pg./ml. with a mean of 347 ± 157 (2 SD). The $UB_{12}BC$ varied from 710 to 2532 pg./ml. with a mean of 1305 ± 672 (2 SD). There was no statistical difference between the values for males and females.

Standard solutions

The *non-radioactive vitamin B_{12} standard* was powdered United States Pharmaceutical grade vitamin B_{12} (Sigma Chemical Company, St. Louis, Missouri, USA) as cyanocobalamin which was diluted in 0.02 M NaH_2PO_4, pH 4.5, containing 0.1 g. potassium cyanide per liter. Standards of 10,000 and 100,000 pg. (10^{-12} g.)/ml. were prepared. The *radioactive vitamin B_{12} standards* were ^{57}cobalt-labelled vitamin B_{12} (^{57}Co-B_{12}) from two lots with specific activities of 140 and 150 μC. per μg. cyanocobalamin (The Radiochemical Centre, Amersham, England). Both preparations contained less than 3 % ^{57}Co not associated with vitamin B_{12} and no corrections were applied. The final standards were prepared by appropriate mixture of the radioactive and non-radioactive standards. The two standards used in the majority of the experiments contained 1830 and 1360 pg. of vitamin B_{12} and 70,000 counts per minute (cpm) in a volume of 50 μl. The vitamin B_{12} content of the standards was checked by microbiological assay using *L. leichmannii* as test organism.

Gel filtration

Gel filtration on Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) was performed as described by Flodin (1962) in 0.1 M Tris buffer, pH 8.00 containing 1 M NaCl using columns 90 to 100 cm. in length with an internal diameter of 2.3 cm. A flow rate of 12 ml. per hour was maintained by a peristaltic pump. Fractions of 3 or 4 ml. were collected by a siphon coupled to a fraction collector. The experiments were carried out at 20 to 22° C.

500 μl. of serum, 50 μl. of the final B_{12} standard and 20 % sucrose were applied to the columns. For the determination of the TC I and TC II binding capacities at

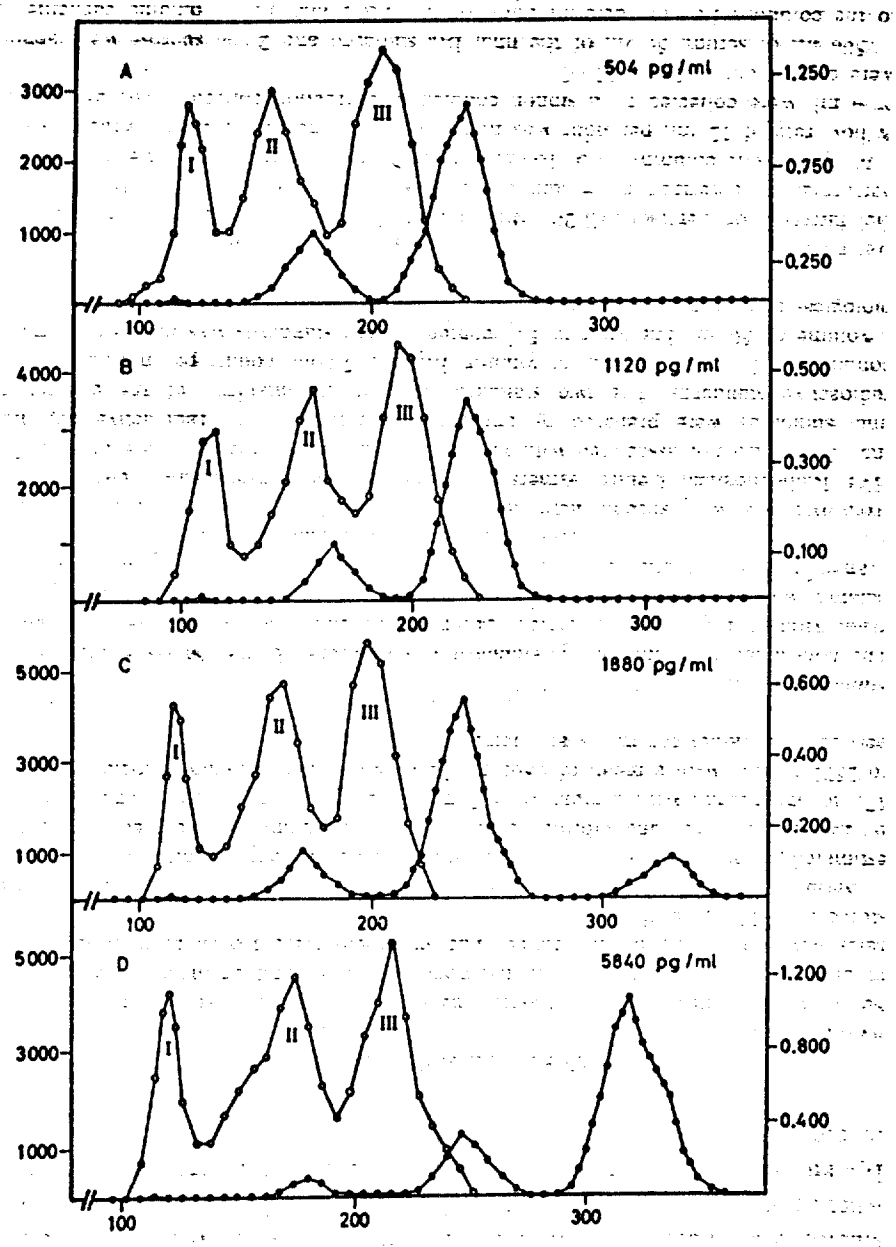

Figure 1. Sephadex G-200 gel filtration of serum from subject K.B.J. (Table I) after addition of different amounts of vitamin B_{12}. Left ordinate, ^{57}Co radioactivity in cpm (●——●). Right ordinate, OD at 280 mμ. (○——○). Abscissa, effluent volume in ml. The relative amount of B_{12} bound to TC I and to TC II remained fairly constant at different levels of added B_{12}.

different levels of added B_{12}, appropriate mixtures of the standards were added to 500 or 1000 μl. of serum.

^{57}Co radioactivity was measured for one minute per effluent fraction in a well-type scintillation counter equipped with a pulse-height analyzer. One μC. ^{57}Co gave 1.8×10^6 cpm with a background of 80 cpm. Since the half-life of ^{57}Co is 267 days, no corrections for radioactive decay were necessary. *Protein content* in the effluent fractions was determined by spectrophotometric measurement at 280 mμ. in a Beckman DU.

RESULTS

Gel filtration separates proteins in decreasing order of molecular size, that is, the larger proteins appear in the effluent before those of smaller size. After addition of an excess quantity of ^{57}Co-B_{12} to normal human serum, three protein peaks (I, II and III), and four radioactive peaks are obtained (Figures 1 and 2). The first radioactive peak, termed TC O, appeared with the high molecular weight proteins of peak I. Labelled TC I appeared between protein peaks II (primarily γG-globulins) and III (albumin), while labelled TC II appeared after protein peak III. The fourth radioactive peak contains non-protein bound ^{57}Co-B_{12} and ^{57}Co not associated with vitamin B_{12}.

The results obtained in an individual serum upon addition of different amounts of vitamin B_{12} are given in Table I. The data demonstrate that, in serum from a particular subject, the relative amount of B_{12} bound to TC I and to TC II remains fairly constant over a range of added B_{12} from 75 to 20,760 pg./ml., although there was a slight increase in the total vitamin B_{12} bound at the higher levels. Eight of the gel filtration diagrams from two subjects are shown in Figures 1 and 2.

Analysis of data of the $UB_{12}BC$ of serum from 50 normal subjects. The frequency distributions of the $UB_{12}BC$ determined by radioisotope dilution and charcoal adsorption, the $UB_{12}BC$ determined by gel filtration and the $UB_{12}BC$ of TC O, I and II are shown in Figure 3. Approximately normal distributions were present for the $UB_{12}BC$ determined by the charcoal method and for the TC II binding capacity, the others showing positively skewed distributions. Transformation to logarithmic values and calculation of the geometric means were therefore carried out assuming a logarithmic normal (lognormal) distribution. Comparison of the arithmetic and geometric means as well as the observed and normal ranges are given in Table II.

Figure 4 compares the $UB_{12}BC$ determined by the charcoal and the gel filtration methods. The correlation coefficient (r) was 0.48. Figure 5 compares the data obtained by gel filtration and gives the results of the analysis of variances obtained after transformation to logarithmic values. The binding capacity of TC I and of TC II was highly significantly correlated to the

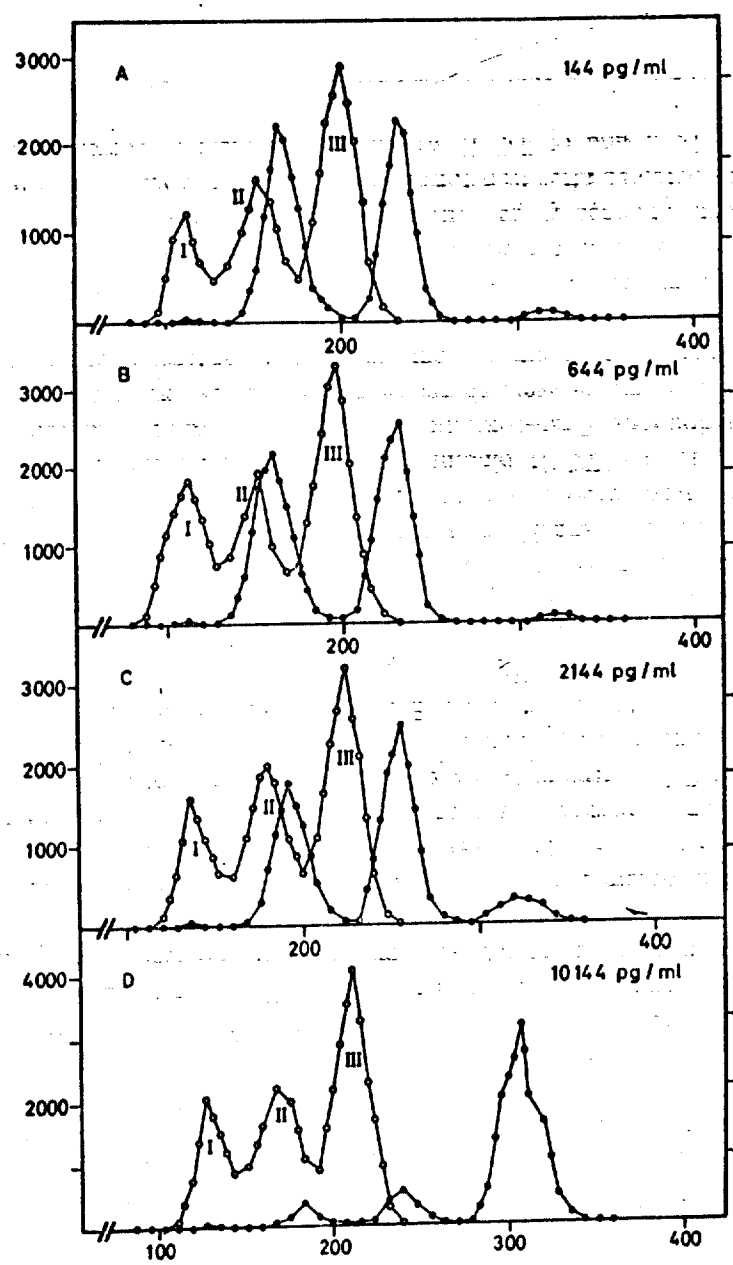

Figure 2. Sephadex G-200 gel filtration of serum from subject L.H. (Table I) after addition of different amounts of vitamin B_{12}. Left ordinate, ^{57}Co radioactivity in cpm (●——●). Right ordinate, OD at 280 mμ. (○——○). Abscissa, effluent volume in ml. The relative amount of B_{12} bound to TC I and to TC II remained fairly constant at different levels of added B_{12}.

TABLE I. *The serum unsaturated vitamin B_{12} binding capacity of TC O, TC I and TC II at different levels of added vitamin B_{12}*

Sephadex G-200 gel filtration data

Subject	Vitamin B_{12} added (pg./ml.)	Radioactivity added (cpm)	Radioactivity recovered (%)	Total B_{12} bound (pg./ml.)	Vitamin B_{12} bound to (pg./ml.)			Vitamin B_{12} bound to (% of total bound)		
					TC O	TC I	TC II	TC O	TC I	TC II
B.K.A.	75	9500	91.1	68	less than 1	21	47	0.3	30.3	69.4
	250	29500	87.2	218	1	81	136	0.3	37.1	62.6
	500	25000	88.5	443	2	122	319	0.5	27.5	72.0
	1000	25000	87.9	879	4	237	638	0.4	27.0	72.6
	2000	46000	90.9	1109	2	339	768	0.2	30.6	69.2
	4000	92500	96.3	1340	15	442	883	1.1	33.0	65.9
B.H.	336	9000	90.6	304	4	66	234	1.3	21.8	76.9
	500	56943	81.2	406	2	86	318	0.4	21.1	78.5
	1172	20000	89.4	1048	4	175	869	0.4	16.7	82.9
	2160	51000	89.9	1326	21	431	874	1.6	32.5	65.9
	10380	17897	90.6	1298	28	254	1016	2.1	19.6	78.3
	20760	61000	86.0	1624	0	338	1286	0	20.8	79.2
K.B.J.	168	16823	98.7	166	1	35	130	0.7	20.9	78.4
	504	37322	89.5	451	2	107	341	0.5	23.8	75.7
	1120	37423	99.5	1114	4	204	906	0.4	18.3	81.3
	1880	53567	93.8	1518	18	270	1230	1.2	17.8	81.0
	2240	71556	92.8	1562	17	277	1268	1.1	17.7	81.2
	5840	59195	88.0	1295	31	262	1002	2.4	20.2	77.4
	10840	59498	99.0	1610	54	279	1277	3.4	17.3	79.3
L.H.	144	25749	91.1	131	0	67	64	0	51.2	48.8
	644	29143	95.4	614	1	310	303	0.2	50.5	49.3
	1144	31102	93.3	1067	3	514	550	0.3	48.2	51.5
	2144	28820	97.5	1856	6	815	1035	0.3	43.9	55.8
	5144	27979	95.0	1954	10	850	1094	0.5	43.5	56.0
	10144	27807	94.2	2027	18	841	1168	0.9	41.5	57.6
G.O.	144	31430	98.4	142	1	39	102	0.7	27.6	71.7
	275	18699	93.5	257	1	71	185	0.4	27.6	72.0
	644	30481	91.9	592	4	170	418	0.7	28.7	70.6
	1150	32481	88.3	1015	5	271	739	0.5	26.7	72.8
	2300	14189	95.2	1019	20	212	787	2.0	20.8	77.2
	5750	32417	95.9	1284	44	187	1053	3.4	14.6	82.0

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TABLE II

Summary of the $UB_{12}BC$ of serum from 50 normal subjects as determined by Sephadex G-200 gel filtration

B_{12} binder	Observed range $UB_{12}BC$		Arithmetic mean pg./ml.	Geometric mean pg./ml.	Normal range* (± 2 SD) pg./ml.
	per cent	pg./ml.			
Total	100	758–2442	1337	1287	751–2206
TC O	0– 5.2	0– 76	21	16	3– 87
TC I	8.5–47.1	72–1057	330	272	79– 939
TC II	52.3–90.3	650–1891	986	959	611–1505

* Based upon lognormal distribution.

total $UB_{12}BC$, the r values being 0.80 and 0.88 respectively. The correlation coefficient between the binding capacity of TC I and that of TC II was 0.43. The binding capacity of TC O was unrelated to the total $UB_{12}BC$ and the binding capacity of TC I and TC II, the r values being -0.13, -0.18 and -0.08 respectively.

DISCUSSION

The results demonstrate a much wider range for the TC I and TC II binding capacities of normal human serum than previously reported. Retief et al. (1967) using a stepwise elution technique through small DEAE cellulose

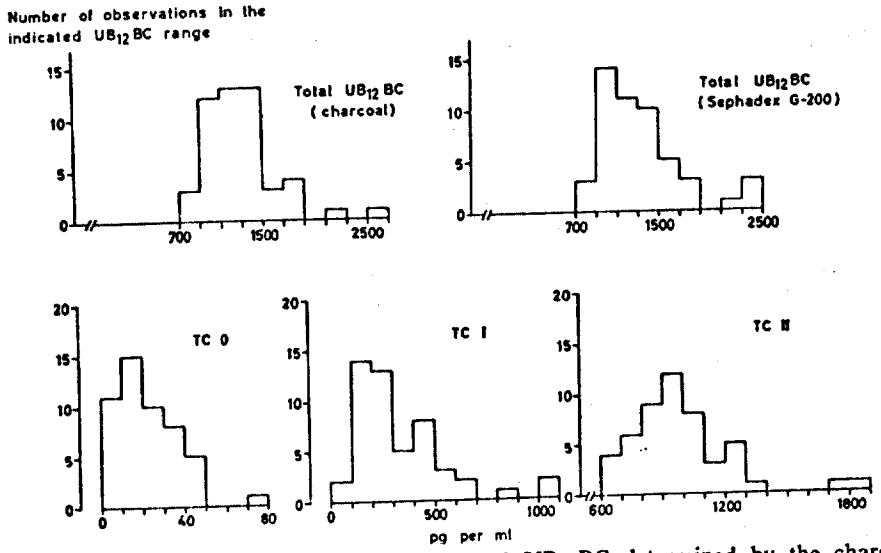

Figure 3. Frequency distributions of the total $UB_{12}BC$ determined by the charcoal adsorption method and the total $UB_{12}BC$, $UB_{12}BC$ of TC O, I and II determined by Sephadex G-200 gel filtration.

columns obtained a range of 12.1 to 29.1 per cent of the $UB_{12}BC$ for TC I (α globulin B_{12} binder) in 5 normal subjects. This range is narrower than the 8.5 to 47.1 per cent TC I reported in the present work for 50 subjects. Mikkonen et al. (1967) using a paper electrophoretic technique, reported a mean ratio of ^{57}Co-B_{12}-TC I to ^{57}Co-B_{12}-TC II of 0.045 $\pm$ 0.026 in sera from 22 control subjects. Conversion of these figures to per cent TC I of the unsaturated TC I and TC II binding capacities results in a mean value of about 4 per cent TC I with a range of about 2 to 7 per cent. The data of Hall & Finkler (1966) are stated to be only semiquantitative due to the difficulty in accurately defining the limits of the transcobalamins in the chromatograms obtained by gradient elution through DEAE cellulose columns. Their data, however, indicate a TC I binding capacity of about 15 to 25 per cent of the TC I and TC II binding capacity, excluding the binding to the main protein peak, after addition of 300 pg. B_{12}/ml. plasma.

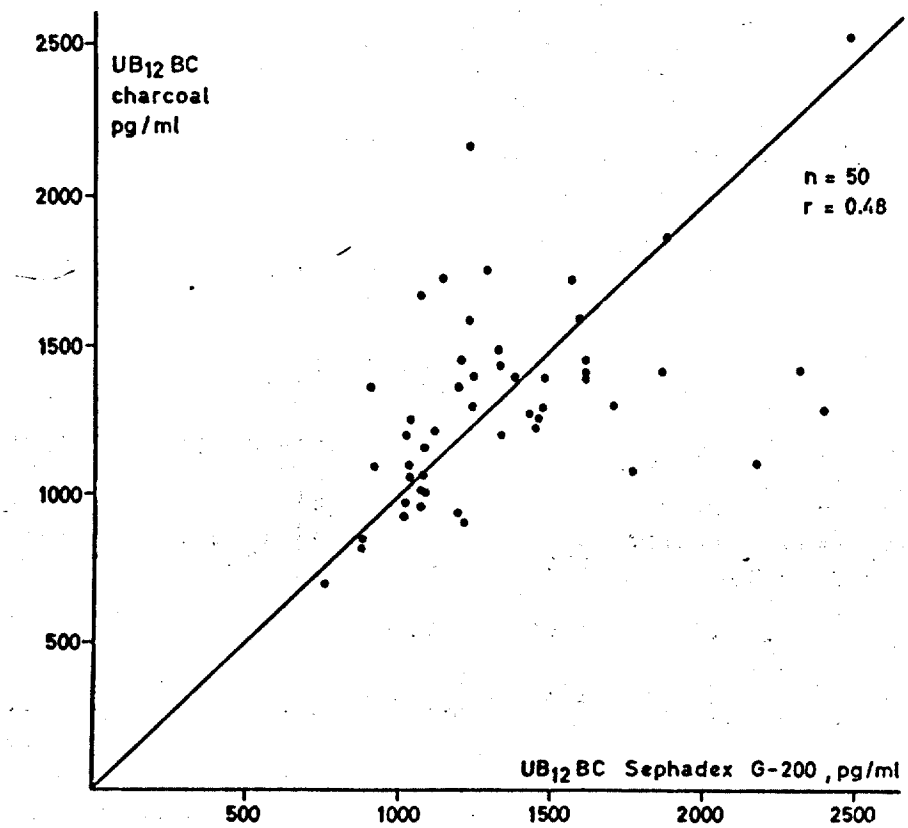

Figure 4. Comparison of the total $UB_{12}BC$ determined by the charcoal and the gel filtration methods. The values were plotted on linear graph paper.

TABLE III

*Data in the literature on the unsaturated vitamin B_{12} binding capacity of TC I of normal human serum and plasma**

Series	Method	Number of subjects	$UB_{12}BC$ of TC I
Hall & Finkler (1966)	DEAE gradient elution	4	about 15–25 **
Retief et al. (1967)	DEAE stepwise elution	5	12.1–29.1***
Mikkonen et al. (1967)	Paper electrophoresis	22	about 2–7 **
Present study	Sephadex G-200 gel filtration	50	8.5–47.1***

* For further details see text.
** Calculated by the present authors and expressed as per cent of the sum of the TC I and TC II binding capacities.
*** Expressed as per cent of $UB_{12}BC$.

Despite the different results obtained in these studies, the important observation is that in normal human serum TC I is not saturated. The results of the data in the literature are summarized in Table III.

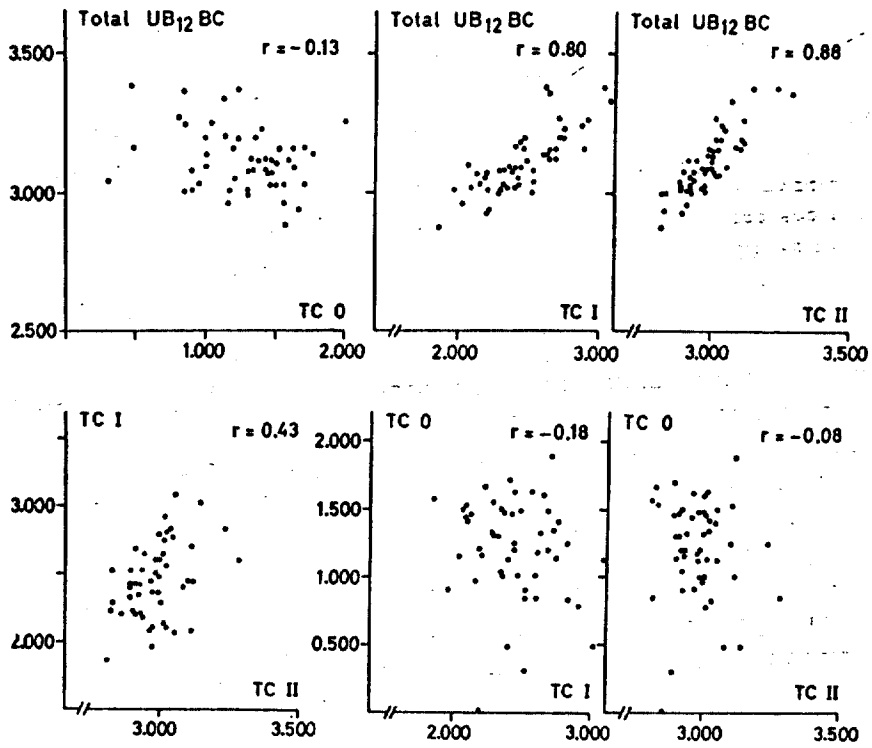

Figure 5. Comparison of the $UB_{12}BC$ of TC O, I and II with each other and with the total $UB_{12}BC$, all determined by Sephadex G-200 gel filtration. Logarithmic values were plotted on linear graph paper (2.000 = 100 pg./ml., 3.000 = 1000 pg./ml.).

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The consistent finding of a high molecular weight vitamin B_{12} binding protein (TC O) has not previously been reported, although suggestions of a third B_{12} binder have been made (Hall & Finkler 1966, Lawrence 1966). The nature of TC O is not known. It may be identical to the TC II complex demonstrable by gel filtration at low ionic strength (Hom 1967), but it may also be a third B_{12} binder present in low concentration in normal serum. TC O, I and II were the only binders of added B_{12} over a range from 75 to 20,760 pg./ml. of serum. Furthermore, the relative amount of B_{12} bound to TC I and to TC II remained fairly constant in an individual serum regardless of the amount of B_{12} added.

The reason for the poor correlation between the $UB_{12}BC$ determined by the charcoal method and the gel filtration method is not known. Because of the size of the haemoglobin, mol. wt. 68,000, used to coat the charcoal, it was thought that variable adsorption of TC II, mol. wt. 38,000 (Hom & Olesen 1967), might explain the discrepant results. This hypothesis was not supported by statistical analysis of the results.

The conclusion that can be drawn from the other correlations is that, in general, the higher the $UB_{12}BC$, the higher the binding capacity of TC I and TC II, indicating that in normal human serum, a high $UB_{12}BC$ is not the result of a selective increase in only one of the B_{12} binders. This is in contrast to patients with chronic myelogenous leukaemia who have been shown to have high $UB_{12}BC$ levels due to increased binding to TC I (Hall & Finkler 1964, 1966) and also to a group of pernicious anaemia patients who possess high $UB_{12}BC$ levels due to the presence of antibodies to TC II (Olesen et al. 1968).

It is apparent that the different results in the literature as well as that reported here are due to the use of different methods. In this laboratory, the gel filtration method for separation of the transcobalamins has been found to be more sensitive, specific and reproducible than electrophoretic, precipitation and ion exchange techniques both in preparative and analytical work. Other considerations, such as speed, availability of equipment and the degree of technical skill required, impose limitations on the use of the gel filtration method on a routine basis.

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Vitamin B_{12} Content of Human Vascular Tissue in Individuals of Various Ages[1]

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VITAMIN B_{12} is a compound which is involved not only in the erythropoietic process but also in the metabolism of proteins and biosynthesis of nucleic acids; its function as cofactor in transmethylation reactions also has been established. Assays of vitamin B_{12} content in human vascular tissue have not previously been reported. The present study was conducted to provide such information.

MATERIALS AND METHODS

The vascular samples were obtained fresh at autopsy from persons at the St. Louis City Morgue who did not display signs of metabolic or infectious diseases. After removal of the adventitia, the intima-media specimens were stored in a freezer. For extraction of vitamin B_{12}, a Kontes Duall grinder was used to homogenize 300 mg. tissue at 4 C. in 6.0 ml. 30 mM aconitate buffer, pH 4.5 as described by Baker and Sobotka (1962). After maintaining at room temperature for 2 hours, the homogenates were steamed for 10 min. and after cooling, filtered through Whatman No. 42 filter paper. The filtrates were diluted 1 : 10 with redistilled water. Preliminary experiments showed that treatment of homogenized vascular tissue with cyanide-activated papain or with diastase at 56 C. for periods extending to 18 hours did not provide better extraction of vitamin B_{12}.

To measure vitamin B_{12}, the microbiological *Ochromonas malhamensis* method was employed with a slight modification of that described by Baker and Sobotka (1962). This procedure was chosen because of its sensitivity and specificity. A 5-day *Ochromonas malhamensis* (ATCC 11532) culture diluted 1 : 10 with sterilized single-strength assay medium was used as inoculum. All vascular samples were analyzed in triplicate at two or three different tissue concentrations: 0.5, 1.0, and 2.0 ml. aliquots of diluted filtrate, corresponding to 2.5, 5.0, and 10.0 mg. fresh tissue, respectively. Double-strength assay medium was prepared and 2.5 ml. was pipetted into 10 ml. micro-Fernbach flasks. After addition of the filtrate aliquot, the volume was made up to 5.0 ml. with redistilled water; the flasks were then stoppered with cotton. After sterilization for 15 min. at 115 C. (12 psi) and subsequent cooling, 0.5 ml. diluted *Ochromonas malhamensis* suspension was added to each test sample. Two flasks with 5.0 ml. single-strength medium were not inoculated; these samples were used to check sterility and served as references for optical density readings.

The flasks were covered with plastic semitransparent sterilized caps and then incubated for 4 days at 28 C. in a thermostat; the incubation was made under constant illumination supplied by two warm-white-fluorescent tubes at a 65 cm. distance. At the end of this period, the content of each flask was diluted by addition of 5.0 ml. redistilled water and shaken. The growth of *Ochromonas malhamensis* was measured turbidimetrically at 540 mμ in a Beckman DU spectrophotometer. Triplicate

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blanks in which the tissue filtrate aliquots were replaced by redistilled water were similarly assayed. A standard curve ranging from 3.9 to 500 $\mu\mu g$ vitamin B_{12} was prepared for each set of tests (Fig. 1). The mean difference between measured vitamin B_{12} tissue concentrations calculated from various quantities of tissue used in the microbiological assays was 5.9%. To check the efficiency of the total analytical procedure outlined in the present paper, separate experiments were conducted in which different amounts of vitamin B_{12} were added to original tissue homogenate samples; quantitative recovery was achieved by subsequent microbiological analyses. Nitrogen determinations were made by the Kjeldahl method (Frankel, 1963) on portions of the same tissue specimens from which the homogenates were prepared.

Results

The mean vitamin B_{12} concentrations in human vascular tissue from Ss of various ages are listed in Table 1. The average content observed for normal aortic tissue (thoracic descending aorta) from adult Ss was 6.39 nanograms/Gm. wet tissue; the corresponding value expressed on the basis of tissue nitrogen content was 157.5 nanograms. Lower levels were obtained for infants and for the 10- to 19-year age group. As seen from Tables 1 and 2, markedly higher vitamin B_{12} contents were found in the pulmonary artery, normal coronary artery, and

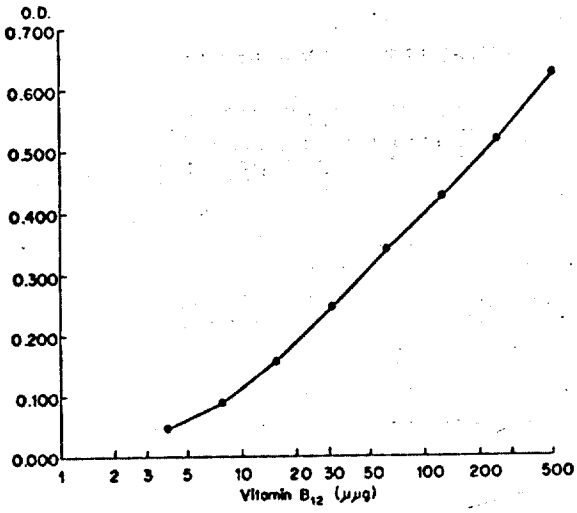

Fig. 1. Standard curve for *Ochromonas malhamensis* procedure. Rate of growth expressed in optical density readings which have been corrected for blank values.

Table 1. Vitamin B_{12} Content of Human Vascular Tissue in Various Age Groups.

Age Group (Years)	N	Mn. Nanograms of Vitamin B_{12}/Gm. Wet Tissue	S.E.	Mn. Nanograms of Vitamin B_{12}/Gm. Tissue Nitrogen	S.E.
Normal Aortic Tissue[a]					
0–1	5	2.30	0.52	61.0	15.5
1–9	4	4.10		99.8	
10–19	5	4.22	0.39	91.0	11.6
20–29	11	6.55	1.01	157.1	22.0
30–39	14	6.18	0.93	153.9	21.9
40–49	8	5.84	1.37	160.8	38.6
50–59	16	5.85	0.63	151.7	16.0
60–69	16	7.00	0.84	192.2	21.5
70–82	4	7.42		207.0	
0–82	83	5.90	0.36	145.0	9.2
20–82	69	6.39	0.41	157.5	10.4
Lipid-Arteriosclerotic Aortic Tissue[a]					
20–29	5	7.58	0.87	183.6	25.2
30–39	4	7.67		201.2	
40–49	4	5.92		160.0	
50–59	9	7.90	0.73	238.2	24.3
60–69	5	6.60	1.34	206.0	42.4
70–82	1	8.90		271.0	
20–82	28	7.33	0.51	207.9	15.2
Fibrous-Arteriosclerotic Aortic Tissue[a]					
40–69	9	4.90	0.90	136.2	24.4
Pulmonary Artery					
20–29	6	16.29	2.97	463.2	96.0
30–39	6	14.50	2.47	418.7	82.0
40–49	4	8.10		246.1	
50–59	10	10.27	0.76	323.3	43.7
60–69	5	14.92	4.10	511.8	158.4
70–82	2	11.50		397.5	
20–82	33	12.63	1.23	389.7	40.3
Normal Coronary Artery Tissue					
20–29	5	13.02	1.96	403.6	47.8
30–39	2	13.35		376.0	
40–49	1	18.50		600.0	
50–59	5	16.32	2.09	527.8	73.6
60–69	1	14.31		482.2	
70–82	1	16.40		587.9	
20–82	15	14.84	1.05	471.9	34.7
Lipid-Arteriosclerotic Coronary Artery Tissue					
40–82	9	9.08	1.19	299.9	4.11
Vena Cava Inferior					
20–29	3	35.70		975.0	
30–39	7	15.87	2.45	486.0	78.6
40–49	6	18.17	3.91	532.0	117.9
50–59	7	23.59	4.19	669.8	115.8
60–69	4	25.08		774.5	
70–82	2	16.45		495.5	
20–82	29	21.60	2.44	613.9	62.0

[a]Thoracic descending aorta

Table 2. Mean Vitamin B_{12} Concentrations of Pulmonary Artery, Normal Coronary Artery, and Vena Cava Inferior Samples Expressed in % of Contents of Normal Aortic Tissue from the Same Subjects.

	N	Wet Tissue	Tissue Nitrogen
Pulmonary artery	32	172.5% $p<0.001$	209.4% $p<0.001$
Coronary artery, normal	14	191.0% $p<0.001$	234.3% $p<0.001$
Vena cava inferior	27	328.5% $p<0.001$	368.9% $p<0.001$

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Table 3. Coefficients of Correlation Between Age and Tissue Vitamin B_{12} Content of Vascular Samples from 20- to 82-year-old Persons.

	N	Wet Tissue r	Tissue Nitrogen r
Aorta normal*	69	+0.04 $p>0.7$	+0.13 $p>0.2$
Aorta, lipid-arteriosclerotic*	28	−0.02 $p>0.9$	+0.22 $p>0.2$
Pulmonary artery	33	−0.12 $p=0.5$	−0.01 $p>0.9$
Coronary artery, normal	15	+0.33 $p>0.2$	+0.45 $p>0.05$
Vena cava inferior	29	−0.03 $p>0.8$	−0.03 $p>0.8$

*Thoracic descending aorta

inferior vena cava specimens than in the aorta. Approximately similar values were recorded for male and female and for white and colored Ss. In the vascular tissues there was no significant variation with age in vitamin B_{12} content for 20- to 82-year-old persons (Table 3).

Although the average vitamin B_{12} values observed for lipid-arteriosclerotic tissue from the 20- to 82-year Ss were higher than those recorded for normal aortic tissue (Table 1), comparison of assays of tissue portions from the same arterial samples (N = 27) did not reveal significant differences. When calculated on the basis of wet tissue weight, the content of arteriosclerotic tissue was 97.2% of that of normal tissue ($p > 0.7$), and when expressed per gram tissue nitrogen 105.8% ($p > 0.4$). In contrast to this, the vitamin B_{12} content was notably lower in arteriosclerotic than in normal coronary artery tissue for the 40- to 82-year age group (wet tissue, 55.7%, $p < 0.01$; tissue nitrogen, 55.6%, $p < 0.01$; $d.f. = 15$).

Discussion

Studies on the vitamin B_{12} content of arterial tissue may be of special importance because it has been reported (Bobkova, 1957) that treatment of arteriosclerotic patients with intramuscular injection of this compound has a beneficial effect. It has further been demonstrated (Zoloeva, Klimov, & Remizov, 1968) that intravenous administration of vitamin B_{12} to rabbits maintained on an atherogenic diet notably prevented the development of arteriosclerosis.

The vitamin B_{12} content of human arterial tissue is of the same order of magnitude as that observed by Girdwood (1952) for a human skin sample and is ten to twenty times higher than values for human serum measured by the *Ochromonas malhamensis* procedure (Baker & Sobotka, 1962). It is of particular interest that whereas most research has shown a great decrease in human serum vitamin B_{12} concentration with age (Boger, Wright, Strickland, Gylfe, & Ciminera, 1955; Chow, Wood, Horonick, & Okuda, 1956; Gaffney, Horonick, Okuda, Meier, Chow, & Shock, 1957; Tauber, Goodhart, Hsu, Blumberg, Kassab, & Chow, 1957) in the present study no significant variation was observed in vascular tissue content of this compound for adult Ss. This is in agreement with approximately similar vitamin B_{12} concentrations reported for liver samples from young and old adults (Swendseid, Hvolboll, Shick, & Halsted, 1957; Pitney & Onesti, 1961; Ståhlberg, Radner, & Nordén, 1967). In this connection it should be mentioned that no significant variation with age in intestinal absorption of vitamin B_{12} has been found (Puddu, Vancini, Rosa, & Barbieri, 1964). It is possible that the vitamin B_{12}-binding capacity to some extent may account for the difference between age changes in serum and tissue.

Summary

Quantitative determinations of vitamin B_{12} concentration in intima-media layers of various human vascular tissues were made by the microbiological *Ochromonas malhamensis* procedure. An average content of 6.39 nanograms/Gm. wet tissue was found in normal aortic samples from adult Ss; lower values were recorded for children. Notably higher vitamin B_{12} values were observed for the pulmonary artery, normal coronary artery, and inferior vena cava than for the aorta. The vitamin B_{12} content of normal and arteriosclerotic portions of aortic samples were of the same order of magnitude, whereas assays of coronary artery specimens revealed lower values in pathological than in normal tissue.

In the various human vascular tissues studied no significant variation with age was found in vitamin B_{12} levels for 20- to 82-year-old persons. This is similar to findings reported for human liver tissue and contrary to the marked decrease in serum vitamin B_{12} concentration which usually has been observed in connection with aging.

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BRITISH MEDICAL JOURNAL

Medical Memoranda

Brit. Med. J. 3:102.

Anaphylactic Reaction after Injection of Vitamin B_{12}

Brit. med. J., 1968, 3, 102

This report concerns a 67-year-old man with pernicious anaemia who developed a severe anaphylactic reaction after receiving an intramuscular injection of vitamin B_{12}, and who showed positive reaction to cyanocobalamin and hydroxocobalamin by intradermal tests.

Case Report

The patient had no history of allergic diseases, and had not previously shown allergic or adverse reactions to drugs. He had been treated for pernicious anaemia since 1949, initially with intramuscular injections of liver extract and cyanocobalamin 0.2 mg. at intervals of two to eight weeks. Cyanocobalamin was given from 1965 to November 1966, when the treatment was changed to hydroxocobalamin. The proprietary preparations of cyanocobalamin and hydroxocobalamin used contained benzyl alcohol 1.5% as preservative, and were distributed in multiple-dose phials.

Until the third and last injection of hydroxocobalamin in April 1967 he had never shown any adverse reaction either to the liver extract or to the different preparations of vitamin B_{12}. Within a couple of minutes after receiving the last injection of hydroxocobalamin he developed symptoms of anaphylactic shock, with peripheral circulatory collapse, unrest, shiverings, and general itching. He had a feeling of swelling of the whole body and was on the point of unconsciousness. After receiving 0.75 ml. of adrenaline subcutaneously he slowly recovered, but had nausea, vomiting, diarrhoea, shiverings, and itching during the following three to four hours, and also developed an evanescent exanthema. During the next couple of weeks he felt unwell and had periods of itching and transitory exanthema.

Results of Intradermal Tests (by Injection)

Test Substance	Test Concentration	Test Dose	Reaction
Cyanocobalamin with 1·5% benzyl alcohol, commercial brand	100 μg./ml.	0·05 ml.	Weal with pseudopodia
Hydroxocobalamin, commercial brand	100 μg./ml.	0·05 ml.	" "
Hydroxocobalamin, very highly purified	100 μg./ml.	0·05 ml.	" "
Cyanocobalamin prepared from very highly purified hydroxocobalamin ..	100 μg./ml.	0·05 ml.	" "
Coenzyme B_{12}	100 μg./ml.	0·05 ml.	" "
Methylcobalamin	100 μg./ml.	0·05 ml.	" "
Benzyl alcohol	1·5%	0·05 ml.	No reaction
Cobalt chloride	10^{-6}	0·05 ml.	No reaction

The results of intradermal tests are shown in the Table. The reactions developed within 10 to 15 minutes. The weals disappeared within four to five hours, while the surrounding flare persisted for about 20 hours. There was no general reaction to the tests. By further dilution of the test substances he gave reactions with weals and pseudopodia even to test concentrations of 1 μg./ml. of all the vitamin B_{12} preparations listed in the Table. He also gave positive reactions to intradermal tests with commercial cyanocobalamin from two other producers of vitamin B_{12}. Intradermal tests with identical technique and test substances were negative in three healthy controls.

The treatment has been changed to a vitamin B_{12} preparation intended for peroral use. So far he has shown no adverse reaction to this medication. It is, however, too early to state if he can utilize the vitamin B_{12} by this route of administration.

Discussion

Reports on anaphylactic shock after injection of vitamin B_{12} preparations have been published by Young *et al.* (1950), Borodulin (1961), Roy (1961), and Marinkovic (1962). In the case described by Roy the anaphylactic reaction resulted in death. Less severe allergic reactions have been reported by Bedford (1952), Gillhespy (1955), and Ungley (1955).

Allergic reactions to proprietary preparations of vitamin B_{12} might be due to substances added to the solutions as preservatives or for other reasons, to impurities from the production, or to the vitamin B_{12} itself. Lagerholm *et al.* (1958) reported a case of hypersensitivity to benzyl alcohol added as preservative, resulting in urticaria after injection.

Vitamin B_{12} is produced by biosynthesis, and the proprietary preparations probably always contain carry-over impurities. Bedford (1952) suggested that these impurities are responsible for the antigenic properties of vitamin B_{12} preparations, and further that there is an antigenic relation between these impurities and antibiotics, and vice versa. A similar explanation is conceivable regarding the case described by Young *et al.* (1950). Their patient developed anaphylactic shock after receiving an injection of vitamin B_{12} concentrate made from streptomyces broth, and gave a positive reaction to intradermal test with this preparation. On the other hand, he neither reacted to intradermal test nor showed evidence of sensitivity to treatment with crystalline vitamin B_{12} made from streptomyces broth.

Animal experiments concerning the sensitizing properties of vitamin B_{12} are controversial. Traina (1950) reported the inability to produce anaphylactic shock in guinea-pigs by multiple injections of small doses of crystalline vitamin B_{12}. On the other hand, Lipton and Steigman (1963), by artificial means, succeeded in sensitizing guinea-pigs to crystalline vitamin B_{12} by intradermal injections of cyanocobalamin emulsified in Freund's adjuvants. They used crystalline cyanocobalamin derived from streptomyces fermentation liquor for sensitization, and the skin reactions were elicited by the use of cyanocobalamin derived from liver. This fact was claimed to ascertain the specificity of sensitization to vitamin B_{12} itself rather than to contaminants in its preparation. Delayed reaction of tuberculin type of crystalline vitamin B_{12} was reported by Rostenberg, jun., and Perkins (1951) in a patient with allergic contact dermatitis from cobalt. They claimed this to be the first report of sensitivity to vitamin B_{12}, and connected the reaction with the cobalt in the vitamin molecule.

The preparations of cyanocobalamin and hydroxocobalamin used in the present patient are highly purified. Though used for years, no reports of untoward reactions have been published. The patient's reactions both to the specially prepared, highly purified hydroxocobalamin and other cobalamins might suggest that he has been sensitized to the cobalamin molecule itself. This should imply that vitamin B_{12} constitutes a complete antigen, an assumption which is not very likely. Further research must decide whether a polypeptide bound to the vitamin, or a carry-over polypeptide, was the antigen in the present case.

My thanks are due to Nyegaard & Co. A/S for the supplies of vitamin B_{12} preparation.

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THE TREATMENT OF HEMORRHAGIC SHOCK WITH CORTISONE AND VITAMIN B_{12}

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USING the dog as the experimental animal, a standard shock preparation has been devised consisting of a slight modification[1] of the methods described by Frank, Seligman, and Fine[2] which produces an exceedingly high mortality within the first 24 to 48 hours of the experiment. Under the conditions of this controlled experiment, cortisone and vitamin B_{12} have been separately administered at various stages to the shocked animals. Their efficacy in the treatment of hemorrhagic shock has then been evaluated by comparison of the respective mortality in the treated and untreated series.

Experience has shown that certain criteria of so-called "irreversible shock" such as the amount of blood withdrawn, the duration of the shock, or the degree of hypotension are not entirely reliable. To be sure, these are all important factors in the mechanism of shock, but the individual ability of the animal to cope with these insults is so variable that they cannot be used as dependably comparable criteria of the extent of shock. For this reason the responsiveness of the individual animal to a standard degree of hypotension or his ability to cope with this insult is considered a more reliable index. Accordingly, the criterion of so-called "irreversibility" is more accurately determined by evidence of collapse of the compensatory mechanism. In this experiment the point of collapse is indicated by the inability of the shocked animal to maintain his blood pressure at the standardized hypotensive level despite repeated small transfusions of blood. Specifically, this point is met when twice within a period of 20 minutes a 25 c.c. blood transfusion fails to maintain the pressure at or above the hypotensive level of 30 mm. Hg.

THE EXPERIMENT

Under Pentothal anesthesia and using sterile technique the femoral vein and artery were cannulated bilaterally. One artery was connected to a manometer to record the blood pressure; the other artery was used to bleed the animal, the blood being collected and heparinized. One vein was used for retransfusion and the other for the gradual administration of a small amount of saline solution (15 c.c.), a vehicle for subsequent drugs. The animal was then bled to a blood pressure of 30 mm. Hg. The rate of bleeding was uniform and determined by the unrestricted flow through a large standard-size cannula. By subsequent small bleedings and later by small transfusions the pressure was maintained at 30 mm. Hg until the time was reached when twice within a period of 20 minutes a 25 c.c. transfusion failed to maintain the pressure at or above 30 mm. Hg.

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Immediately thereafter, all of the remaining collected blood was reinfused and the animal was given 300,000 units of penicillin intramuscularly. Heparin, 2.5 mg. per 100 c.c. of blood, was used as the anticoagulant.

Control Series.—Fifteen dogs were used as controls during this experiment in addition to 20 dogs in a previous study of this kind in our laboratory.[3] The shock pattern was uniform. At the completion of the experiment, with reinfusion of the remaining blood, the blood pressure would return almost to its normal level (approximately 120 mm. Hg) (Fig. 1). Periodic blood pressure

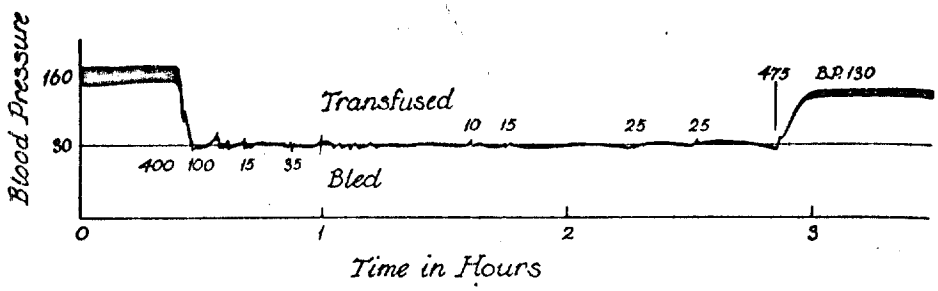

Fig. 1.—Hemorrhagic shock. Protocol of a control experiment. Note the rise in blood pressure during the final transfusion.

determinations after the completion of the experiment showed that the rise in blood pressure after retransfusion was usually transient, lasting only for several hours and gradually falling until the animal died (Fig. 2). All of the animals in this control series of 15 dogs were dead within 24 to 48 hours and 15 of the 20 dogs in the previous control series died within this period, giving a mortality of 86 per cent for the combined control series (Table I).

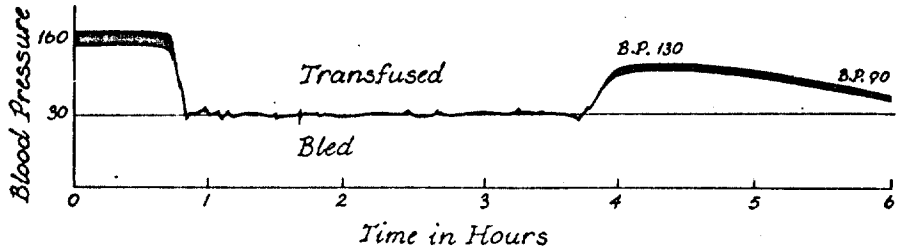

Fig. 2.—Hemorrhagic shock. Protocol of a control experiment demonstrating the secondary fall in blood pressure following the final transfusion.

Cortisone.—Because adrenal cortical secretion appears to be a physiologic response to stress, the following experiment was performed in an effort to determine the value of cortisone in carrying the animal through an otherwise fatal insult from hemorrhagic shock.

Using 50 animals, the experiment as previously described was repeated except that 50 to 200 mg. of cortisone (Cortone*) were given intramuscularly. The cortisone was administered usually as a single injection at variable times during the experiment, from 48 hours prior to the shock procedure to immediately after the retransfusion of the animal.

*Provided through the courtesy of Merck & Company, Inc., Rahway, N. J.

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TABLE I. CONTROL SERIES

	NUMBER DOGS	DIED WITHIN 48 HR. NUMBER	PER CENT
Controls (present series)	15	15	100
Controls (previous series)	20	15	75
Total	35	30	86

Results of Cortisone Therapy.—The 48 hour mortality for the entire treated series is 82 per cent, which appears somewhat lower than the 100 per cent for the control series in this experiment (Table II). When compared, however, with the mortality of 86 per cent for the combined control series, it is apparent that no significant difference between these series exists. It is also apparent that the cortisone had no effect whether administered before, during, or after the shock procedure (Table II). Further analysis revealed that the treated animals that lived were not exposed to shock as long nor bled as severely as those treated animals which died, suggesting that factors other than therapy may have played a role in their survival (Table III).

TABLE II. RESULTS OF CORTISONE THERAPY

TIME OF ADMINISTRATION	NUMBER DOGS	DIED WITHIN 48 HR. NUMBER	PER CENT
Preoperatively	24	20	83
During shock	7	6	86
After retransfusion	19	15	79
Total	50	41	82

TABLE III. HEMORRHAGIC SHOCK; RESULTS OF CORTISONE THERAPY

	NUMBER DOGS	AVERAGE WEIGHT (KILOGRAMS)	AVERAGE TIME IN SHOCK (MIN.)	AVERAGE HEMORRHAGE PER KILOGRAM OF BODY WEIGHT (C.C.)
Controls	15	14.7	132	38.9
Treated, lived	9	15.4	95	39.1
Died	41	14.6	129	49.1

Since cortisone appears to have little or no value in altering the end results of this type of shock, the question arises as to whether or not it has any effect upon the shock pattern. This was determined by a comparison of 2 essential factors, duration of shock and extent of hemorrhage, among the animals receiving cortisone prior to shock and those not receiving it at this time. It is apparent from an analysis of the data in this respect that cortisone did not significantly alter the shock pattern (Table IV).

TABLE IV. EFFECT OF PREOPERATIVE CORTISONE ON SHOCK PATTERN

	NUMBER DOGS	AVERAGE TIME IN SHOCK (MIN.)	HEMORRHAGE PER KILOGRAM OF BODY WEIGHT (C.C.)
Preshock cortisone	22	127	43.0
No preshock cortisone	27	121	44.4

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Vitamin B_{12}.—Certain observations suggested the possibility that vitamin B_{12} might have some value in this form of shock. For one thing, vitamin B_{12} is a crystalline liver substance and evidence has been presented that, at least in the experimental animal, the liver plays a significant role in the mechanism of hemorrhagic shock.[1-4] For another, vitamin B_{12} has shown promise in the treatment of anaphylatic shock.[5] For these reasons, it was considered desirable to test its efficacy in the treatment of hemorrhagic shock under the conditions of this experiment. The dosage employed is massive when compared to the therapeutic dose used in pernicious anemia.

TABLE V. RESULTS OF VITAMIN B_{12} THERAPY (900 μG.)

TIME OF ADMINISTRATION	NUMBER DOGS	DIED WITHIN 48 HR.	
		NUMBER	PER CENT
During shock procedure	21	17	81
After completing shock procedure	7	6	86
Total	28	23	82

The experiment was, therefore, repeated using 28 dogs. Each animal was given intravenously 900 μg. of vitamin B_{12} (Cobione*) in 15 c.c. saline solution. The drug was given at the rate of 75 μg. per 10 minute period either during the shock procedure or at the completion of the experiment following the retransfusion. It was found that if the drug was given rapidly a fall in blood pressure occurred, so that in order to avoid influencing the criteria for terminating the experiment 7 animals were given the drug only at the completion of the shock procedure.

TABLE VI. HEMORRHAGIC SHOCK; RESULTS OF VITAMIN B_{12} THERAPY

	NUMBER DOGS	AVERAGE WEIGHT (KILOGRAMS)	AVERAGE TIME IN SHOCK (MIN.)	AVERAGE HEMORRHAGE PER KILOGRAM OF BODY WEIGHT (C.C.)
Controls	15	14.7	132	38.9
Treated, lived	5	17.3	112	54.7
Died	23	15.9	119	45.2

Results of Vitamin B_{12} Therapy.—A comparison of the 48 hour mortality among the animals receiving vitamin B_{12} during the shock procedure with the mortality of those receiving the drug after completion of the experiment revealed no significant differences (Table V). Further analysis of the data showed that the shock pattern as reflected by the duration of shock and the extent of

TABLE VII

	NUMBER DOGS	DIED WITHIN 48 HR.	
		NUMBER	PER CENT
Controls	35	30	86
Cortisone	50	41	82
Vitamin B_{12}	28	23	82

*Provided through the courtesy of Merck & Company, Inc., Rahway, N. J.

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hemorrhage was not significantly altered among the treated animals that lived and those that died (Table VI). It is also apparent that the mortality among the treated animals is not significantly different from that of the controls, the respective figures being 82 per cent and 86 per cent (Table VII).

SUMMARY

1. A method of producing a highly lethal form of hemorrhagic shock is described. This consists essentially in bleeding the animal until the systolic pressure reaches 30 mm. Hg and maintaining it at this level by subsequent small bleedings or reinfusion of blood until a specific criterion of collapse of the compensatory mechanism is evident. This criterion is met when twice within a period of 20 minutes a 25 c.c. blood transfusion fails to maintain the pressure at or above 30 mm. Hg. In the combined control series of animals this type of hemorrhagic shock produced a mortality of 86 per cent (Table VII).

2. Using 50 dogs the experiment was repeated except that 50 to 200 mg. of cortisone were given intramuscularly before, during, or after the completion of the experiment. Eighty-two per cent of the animals so treated died within 48 hours and only 10 per cent of the animals completely recovered from the experiment (Table VII).

3. An additional 28 dogs were used in a similar experiment to test the efficacy of vitamin B_{12} in the treatment of this type of hemorrhagic shock. Eighty-two per cent of the animals so treated died within 48 hours and 18 per cent recovered from the experiment (Table VII).

4. The shock pattern as reflected by duration of shock and extent of hemorrhage was not significantly altered by either cortisone or vitamin B_{12}.

5. These results, therefore, indicate that neither cortisone nor vitamin B_{12} has any significant effect upon the course of hemorrhagic shock under the conditions of this experiment.

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ARTICLES

VITAMIN B_{12}-DEPENDENT METHYLMALONICACIDURIA: AMINO ACID TOXICITY, LONG CHAIN KETONURIA, AND PROTECTIVE EFFECT OF VITAMIN B_{12}

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ABSTRACT. Methylmalonicaciduria is an inborn error of metabolism characterized by protein intolerance, neonatal or infantile ketoacidosis, long chain ketonuria, and by the accumulation of large amounts of methylmalonic acid (MMA) in blood and urine. The present study examines the metabolic basis for these features in a young boy with the vitamin B_{12}-dependent variant of this disorder. A diet containing 5 gm of protein per kg body weight per day resulted in transient ketoacidosis, long chain ketonuria, and increased methylmalonicaciduria. Isoleucine, valine, methionine, and threonine, all known MMA precursors, were responsible for this protein intolerance. Oral loads of each of these amino acids resulted in equimolar 1,000 μg increases in urinary MMA. Valine and isoleucine also precipitated mild ketoacidosis and long chain ketonuria.

Injections of vitamin B_{12} (1000 μg per day) promptly lowered urine MMA by 70% and partially corrected the defect in propionate or methylmalonate oxidation found in the patient's leucocytes or platelets. While receiving vitamin B_{12}, the clinical toxicity of a valine load was abolished and its biochemical effects significantly modified.

These findings demonstrate that the observed protein intolerance and ketoacidosis in patients with methylmalonicaciduria are direct results of the primary block in methylmalonate catabolism and that vitamin B_{12} acts by overcoming, in part, this metabolic block. These conclusions imply that children with this disorder should respond to dietary protein restriction and/or vitamin B_{12} supplementation, a regimen on which our patient has thrived for over 2 years. *Pediatrics*, 46:497, 1970.

METHYLMALONICACIDURIA, INBORN ERRORS, VITAMIN B_{12}, KETONURIA.

EXCESSIVE methylmalonic acid (MMA) excretion in human urine is diagnostic of either vitamin B_{12} (B_{12}) deficiency,[1] or of a recently recognized inborn error of metabolism, methylmalonicaciduria.[2] Infants with the latter metabolic disorder have vomiting, severe metabolic ketoacidosis, and may succumb to overwhelming infection. They have marked protein intolerance,[2-6] and may have severe growth retardation. Since 1967, 13 patients with congenital methylmalonicaciduria or methylmalonicacidemia have been reported from several medical centers in Europe[2,3,7] and North America,[4,8,9] suggesting that this condition may be an important cause of life-threatening ketoacidosis in infancy. Two forms of this condition have been identified by the response to B_{12} injections. Although none of these patients has had B_{12} deficiency, vitamin B_{12} dependency[10] has been demonstrated in five children whose urinary MMA levels decreased markedly after large doses of B_{12}.[4,6,9,11,12] Several other patients have failed to show any response to B_{12}.[3,5,8]

The present investigations in a patient with the vitamin B_{12}-dependent variant of methylmalonicaciduria, detail the biochemical and clinical evidence that protein intolerance is caused by those amino acids which are precursors of MMA, and that two

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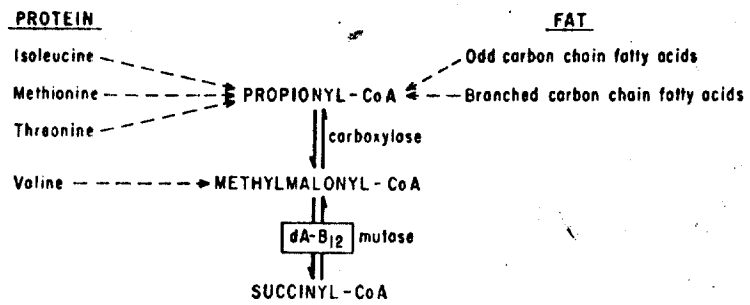

FIG. 1. Biochemical pathway of methylmalonate biosynthesis and catabolism. The defect in methylmalonicaciduria is at the step catalyzed by the mutase apoenzyme and dA-B_{12}.

of these amino acids are precursors of the long chain ketones characteristic of this disorder. Finally, data are presented which demonstrate that vitamin B_{12} modifies both the clinical and biochemical consequences of this metabolic disorder.

CASE REPORT

R.P. is the boy reported previously by Rosenberg, *et al.*[4] At 3 months of age he had mucus diarrhea and vomiting, and became dehydrated and lethargic. On admission to another hospital he had grunting respirations due to severe metabolic acidosis (blood pH 7.15; serum bicarbonate 6 mEq/L). The acidosis cleared slowly with intravenous fluids containing sodium lactate. At the age of 8 months he was admitted to Yale-New Haven Hospital with a similar acute attack of grunting respirations. He arrived comatose with severe ketoacidosis which responded to vigorous intravenous fluid therapy. As previously reported, he had methylmalonicaciduria and methylmalonicacidemia, intermittent hyperglycinemia, transient leukopenia and thrombocytopenia, and excreted long chain ketones when ketotic.[4] Repeated analyses of plasma and urine revealed normal concentrations of methionine and no homocystine or cystathionine. His methylmalonicaciduria decreased dramatically with large doses of cyanocobalamin (CN-B_{12}).[11] Since the earlier report, he has thrived on a low protein diet (2 to 3 gm per kilogram body weight per day), and supplementary B_{12} injections (1000 μg twice weekly). At the age of 27 months, his weight and height were within normal limits: 11.45 kg and 85 cm.

His intellectual development has been assessed periodically by Doctor M. F. Leonard,* using the Yale Child Study Center Revised Developmental Schedules. When first seen his developmental quotient was only 50% but has improved progressively to 90% at the age of 24 months, when he succeeded in all nonverbal tasks at the 18-month level, and achieved the 30-month level in some tasks.

A sister was born in October, 1968. Amniotic fluid obtained in the thirty-sixth week of gestation had no detectable MMA, cord blood leucocytes oxidized radioactive propionate, methylmalonate and succinate normally, and she excreted no MMA in her urine. She is thriving on a normal diet, and her peripheral blood platelets and leucocytes continue to show normal oxidation of propionate, methylmalonate and succinate.

BIOCHEMICAL MECHANISMS

Methylmalonate, as its coenzyme A (CoA) ester, is the intermediate between propionate and succinate in the major mammalian catabolic pathway for propionate and its precursors (Fig. 1). Propionyl-CoA is carboxylated to D-methylmalonyl-CoA by propionyl-CoA carboxylase (E.C. 6.4.1.3). A specific racemase (E.C.5.1.99.1) converts the D-stereo-isomer to L-methylmalonyl-CoA.[13] L-methylmalonyl CoA is then isomerised to succinyl CoA by L-methylmalonyl-CoA carbonylmutase (mutase, E.C.5.4.99.2). This mutase is the only mammalian enzyme known to require 5'-deoxy-adenosyl cobalamin (dA-B_{12}), one of the coenzyme forms of B_{12}, for its action.[14] The metabolic block in methylmalonicaciduria is at this mutase reaction.

The amino acid, valine, and the pyrimidine, thymine, are catabolized via methylmalonyl-CoA. Isoleucine, methionine, and threonine are catabolized via propionyl-

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CoA, as are the branched-carbon chain and odd-numbered-carbon chain fatty acids and cholesterol. Leucine does not enter the propionate pathway at all.

METABOLIC STUDIES

Dietary Stress

Dietary protein was increased from 1 gm per kg body weight per day (kg/day) to 2, 3, and 5 gm/kg/day at 5-day intervals. Serial 24-hour urine samples were assayed for MMA and creatinine. Each urine specimen was tested for the presence of ketone bodies. The 5 gm protein diet was stopped after 3 days because the patient was vomiting, developed ketonuria, and became mildly acidotic (serum bicarbonate 18 mEq/L).

Amino acid loading tests were conducted with valine, leucine, isoleucine, methionine, and threonine, while the patient was receiving 3 gm protein/kg/day. An amount of each amino acid equivalent to that contained in 5 gm casein/kg/day was given orally in four divided doses for 5 days, after at least 5 control days on the basal diet alone (3 days for threonine). Valine loading was stopped after 3 days and isoleucine was stopped after 3½ days because of vomiting, ketonuria, and onset of acidosis. The effects of a 24-hour fast were also observed and compared with those of the dietary loading tests.

Vitamin Dependency

The response to B_{12} was measured by comparing urine MMA output for 5 control days while the patient was given a basal diet of 3 gm protein/kg/day, with urine MMA while the patient was receiving 1,000 μg CN-B_{12} daily in single or multiple intramuscular injections. The minimal effective dose of CN-B_{12} was determined by giving daily injections of 5 μg for 4 days, then increasing the dose successively at 4-day intervals to 15 μg and 200 μg. Larger doses were also tested by giving four injections of 1,000 μg each day for 8 days and then 10,000 μg orally per day for 4 weeks. The coenzyme form of vitamin B_{12}, dA-B_{12}, (1,000 μg daily by injection) was also tested for its effect on urine MMA output.

To compare the clinical and biochemical response to valine loading with and without B_{12}, the following was done: after stabilization on a diet of 3 gm protein/kg/day, an oral valine load was given for 3 days as be-

TABLE I

TOXIC EFFECTS OF DIETARY PROTEIN AND AMINO ACIDS

Body Weight	*Intake*	*Days Given*	*Amino Acid Load* (*per kg body weight*)		*Total Urine MMA Increase**	*Clinical Effect*	
Kg	(*per kg body weight per day*)		*mmole per day*	*mmole total*	(*mmole/kg body wt.*)	*Vomiting*	*Ketonuria*
5.8	5 gm protein	3	2.8†	8.4†	>2.7††	++	++++
6.4	24-hour fast	1	--	--	−1.4	++	++++
6.0	3 gm protein+valine	3	3.1	9.3	>6.1††	++	++++
6.1	3 gm protein+leucine	5	3.6	18.0	0	-	±
6.2	3 gm protein+isoleucine	3½	2.5	8.6	>4.9††	++	++++
6.5	3 gm protein+methionine	6	1.0	6.1	6.1	±	+
6.8	3 gm protein+threonine	5	1.8	9.1	9.5	-	++
9.6	3 gm protein+valine	3	3.1	9.3	9.9	++	++++
10.1	3 gm protein+valine (On vitamin B_{12} 1,000 μg/d)	3	3.1	9.3	3.6	-	-

* Total amount of urine MMA excreted in excess of basal output during each study period.

† Estimated load of all toxic amino acids in 2 gm casein.

†† Incomplete urine collection.

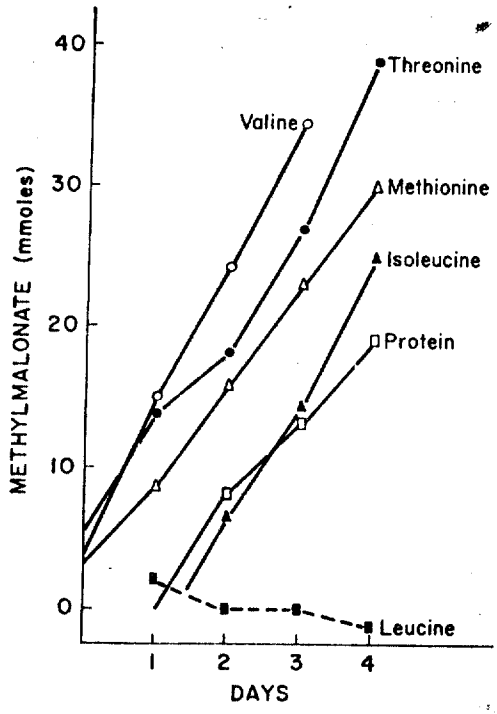

FIG. 2. Effect of dietary protein and amino acids on urinary methylmalonate. Daily methylmalonate excretion in excess of R.P.'s control values is cumulated for each dietary load. See text and Table I for details.

fore: 7 days after completion of the valine load, daily injections of CN-B_{12} (1,000 μg) were started, the valine load was then repeated on the fourth to seventh day of CN-B_{12} injections, which were continued while MMA output was measured from the fourth to tenth days.

METHODS

Urine MMA was measured by our modification[11] of the colorimetric method of Giorgio and Plaut.[15] Whenever there was significant ketonuria, neutral ketones were extracted as their dinitrophenylhydrazones and identified by thin layer chromatography with hexane-chloroform.[4] *In vitro* tests of leukocyte oxidation of radioactive propionate, methylmalonate, and succinate were carried out as described previously.[11,16] Platelet-rich plasma was obtained from fasting heparinized blood by centrifuging at 160 × g for 10 minutes at room temperature, and platelet-poor plasma by centrifuging at 300 × g for 20 minutes. Zero point nine of platelet-rich plasma of leukocyte suspension was incubated with 0.1 ml of radioactive propionate (1.65 μmole) methylmalonate (1.65 μmole) or succinate (1.65 μmole) for 3 hours at 37°C. and radioactive carbon dioxide was collected at 4°C and measured as described previously.[16] When dA-B_{12} solutions, which are unstable to light were used, they were prepared in a dark room, and light was rigorously excluded from the reaction mixture during incubation.†

RESULTS

Effect of Age on Excretion of MMA

There was a definite decrease in MMA output with age. At 10 months of age, while on a diet containing 3 gm protein/kg/day, urine MMA was 0.93 ± 0.13 mmoles/kg/day (mean ± 1 S.D.). By age 24 months, urinary MMA had fallen to 0.57 ± 0.09 mmoles/kg/day on the same diet.

Effect of Diet

On diets containing 1, 2, and 3 gm protein/kg/day the patient was asymptomatic, but ingestion of 5 gm protein/kg/day caused vomiting and ketonuria by the second day and acidosis by the third day. Dietary protein was then reduced to 2 gm/kg/day, and the acidosis and ketonuria cleared within 1 day. Urinary MMA doubled during loading with 5 gm protein/kg/day. A 24-hour fast, which also caused vomiting and ketonuria, was followed by a fall in urinary MMA to 0.25 and 0.35 mmoles/kg/day for the 2 following days (Table I).

† Polyvinylpyrollidone was purchased from Sigma Chemical Corporation, 3500 De Kalb Street, St. Louis, Missouri 63118. The radioactive chemicals were purchased from New England Nuclear Corporation, 575 Albany Street, Boston, Massachusetts 02118.

The dA-B_{12} was received as gifts from Doctor W. L. Koeber of the Squibb Institute for Medical Research, and from Doctor R. H. Abeles of Brandeis University.

Amino Acid Loading

Each of the amino acids tested, with the exception of leucine, caused a marked increase in urinary MMA, and varying degrees of ketonuria (Fig. 2). Valine resulted in a threefold increase in urinary MMA; ketonuria appeared on the second day of the loading test, was severe on the third day, and disappeared within 24 hours of stopping the oral valine. Urinary MMA, however, remained elevated for 3 more days. The total increased excretion of MMA, in excess of basal output, approximated the total amount of valine given (Table I). Isoleucine raised urinary MMA to 2.8 mmoles/kg/day and caused ketonuria while the amino acid was being given. The total increment in MMA output produced by isoleucine loading was not determined since urine collections were discontinued after the last day of isoleucine administration. Methionine and threonine caused no clinical disturbance, but slight ketonuria appeared on the fifth day of the loading study. The total ingested load of methionine and threonine was equalled by the total excess

TABLE II

EFFECT OF DIETARY LOADING TESTS ON FRACTIONATION OF NEUTRAL URINARY KETONES

Diet (per kg body weight)	*Per Cent of Total Neutral Dinitrophenylhydrazones*		
	Acetone	*Butanone*	*Hexanone*
24-hour fast	91.4	7.5	1.1
5 gm protein	80.7	16.2	3.1
3 gm protein + valine	66.2	23.5	10.3
3 gm protein + isoleucine	23.9	49.4	26.7

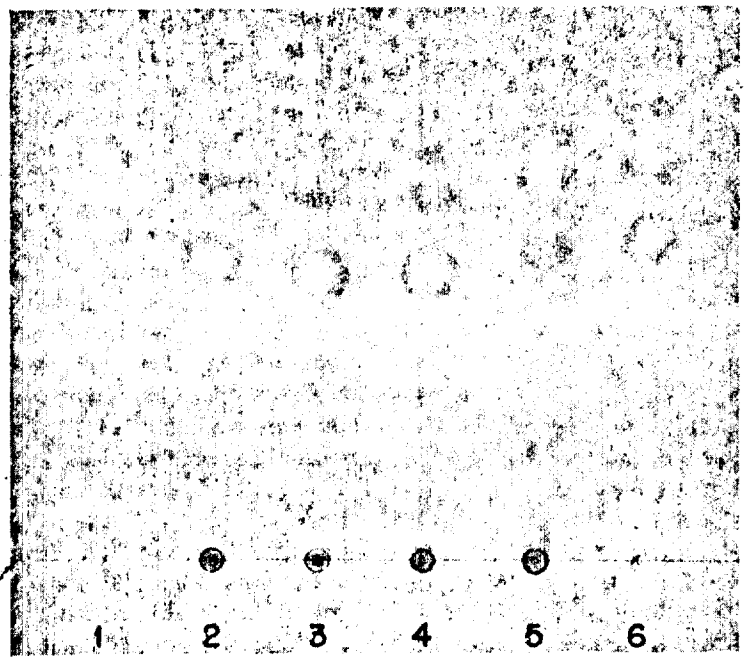

FIG. 3. Photograph of silica gel thin layer chromatogram of neutral urinary dinitrophenylhydrazones from R.P. The following hydrazones were spotted in the numbered channels: (1) acetone and butanone (acetone is closest to the origin); (2) urine obtained on 5 gm/kg protein diet; (3) urine obtained during 24-hour fast; (4) urine while receiving an oral load of L-valine; (5) urine while receiving an oral load of L-isoleucine; (6) acetone, butanone and l-hexanone standards. See Table II for quantitative results.

TABLE III

OXIDATION OF PROPIONATE AND SUCCINATE BY BLOOD PLATELETS

Subjects	$^{14}CO_2$ Produced From	
	Propionate-3-^{14}C (1.65 μmole/ml)	Succinate-1,4-^{14}C (1.65 μmole/ml)
Controls	14.2±4.7† (6)	44.5±17.1 (5)
Controls+B_{12}*	10.3±0.8 (7)	
R.P.	1.0±0.7 (4)	48.8±17.1 (4)
R.P.+B_{12}*	0.6±0.2 (5)	
R.P. while receiving B_{12} injections	3.8	25.4

* Addition of 10 to 50 μg of dA-B_{12} or CN-B_{12} to incubation mixture.

† Nanomoles $^{14}CO_2$/3 hour/10^{10} platelets. The data are presented as the mean±S.D. and the number of observations is shown in parenthesis.

of MMA output. In contrast to these results, large amounts of leucine given for 5 days failed to produce any rise in urinary MMA and produced only minimal ketonuria.

Long Chain Ketones

The pattern of neutral ketones excreted in the urine was directly related to the cause of the ketosis (Table II and Fig. 3). When ketosis was induced by a 24-hour fast, more than 90% of the neutral ketones excreted was acetone. When the patient was made ketotic by ingesting a diet containing 5 gm protein/kg/day, 80% of the neutral ketones excreted was acetone, over 15% was butanone, and 3% was hexanone. The butanone and hexanone fractions increased sharply when ketosis was produced by oral loading with either valine or isoleucine. With valine 24% of the neutral ketones was butanone, and 10% was hexanone. With isoleucine, 49% was butanone, 27% was hexanone, and 24% was acetone.

In Vitro Studies

Previous studies with peripheral blood leukocytes and cultured fibroblasts from R.P. had demonstrated a defect in methylmalonate conversion to succinate.[11,17] In these cells, oxidation of propionate and methymalonate was virtually absent whereas oxidation of succinate was normal localizing the defect to the mutase reaction. Platelets have been found to demonstrate this defect (Table III).

Addition of 1,000 to 10,000 ng/ml of CN-B_{12} or dA-B_{12} to the incubation mixture failed to stimulate the defective oxidation of methylmalonate by intact leucocytes or platelets from R.P. Pre-incubation of whole blood with 1,000 ng/ml CN-B_{12} or dA-B_{12} *in vitro* for 24 hours also failed to improve the defect in the patient's leucocytes.

Response to Vitamin B_{12} Administration

We showed previously that parenteral administration of CN-B_{12} resulted in a prompt decline in urinary MMA and a concomitant increase in oxidation of radioactive propionate or methylmalonate by peripheral blood leucocytes.[11] These observations were confirmed repeatedly in our patient over the course of 12 months. Neither response, however, reached normal values. When injections of CN-B_{12} (1,000 μg) were given daily, urinary MMA fell to 0.31 mmoles/kg/day but the patient still excreted 300 mg of MMA per 24 hours, a value far in excess of the upper limit of normal (< 5 mg/day for adults). When this daily dose of CN-B_{12} was divided into four injections, no further reduction in urinary MMA was noted. Increasing the dose to 4,000 μg per day lowered urinary MMA only slightly more to 0.2 mmoles/Kg/day. CN-B_{12} in smaller daily doses from 5 μg to 200 μg resulted in little if any reduction in urinary MMA. Since a Schilling test in R.P. had shown that 10% of an oral dose of ^{60}Co-labelled CN-B_{12} was absorbed, he was given 10,000 μg of CN-B_{12} orally, daily for 4 weeks. On this regimen his urinary MMA also fell to 0.3 mmoles/Kg/day. Injections of dA-B_{12} (1,000 μg) daily reduced urinary MMA to 0.2 mmole/Kg/day.

Although CN-B_{12} only partially correcte

the methylmalonicaciduria, it completely abolished the toxic effects of valine loading. When the patient was given the valine load while receiving CN-B_{12} injections, no vomiting or acidosis occurred and merely a trace of ketones was detectable in the urine at the end of the second day. Without B_{12}, plasma lactate rose to 48 mg/100 ml (5.1 mM) 4 hours after the last oral dose of valine, but with B_{12} lactate increased to only 20 mg/100 ml (2.1 mM). Urinary MMA also rose much less when the patient was receiving the vitamin (Fig. 4) and returned to basal levels promptly at the end of the loading period, in contrast to the persistent elevation observed without B_{12}.

Oxidation of propionate and methylmalonate by R.P.'s leucocytes also improved while he was being given B_{12}. This improvement was not seen until more than 24 hours after the first injection of dA-B_{12} (Fig. 5) but methylmalonate oxidation then rose to near normal levels and remained up for more than 2 weeks after the last injection of dA-B_{12}. This effect of B_{12} injections was also evident in the patient's platelets (Table III).

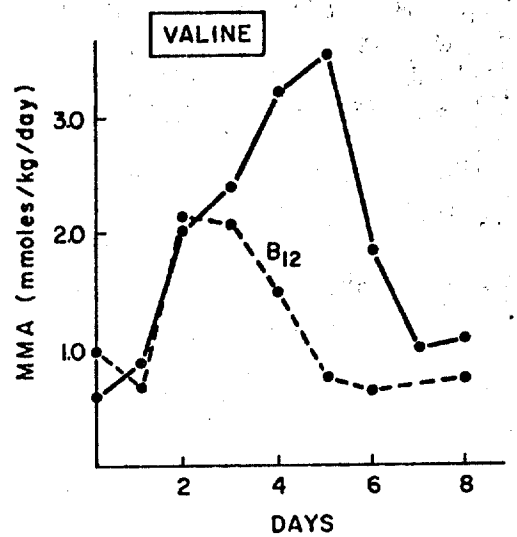

FIG. 4. Urine methylmalonate excretion in response to a 3-day valine load. The continuous line represents the response without vitamin B_{12}, and the broken line represents the response while R.P. was receiving 1,000 μg of cyanocobalamin daily by injection.

Family Study

Repeated tests of the patient's parents failed to demonstrate any evidence of defective methylmalonate catabolism in leukocytes or platelets. Varying the duration of incubation or the number of cells used, or increasing propionate concentration from 1.65 mM to 16.5 mM failed to distinguish these postulated heterozygous carriers from normal individuals.

DISCUSSION

Biochemical Basis of Protein Intolerance and Ketoacidosis

Urinary MMA gave a precise measure of the biochemical response to dietary stress in R.P. Fasting was followed by a sharp drop in MMA excretion suggesting that a major portion of urinary MMA was derived from dietary sources. In contrast, a high protein diet (5 gm/kg/day) or corresponding increases of amino acid precursors of MMA (Fig. 1) caused a distinct rise in urinary MMA and caused toxicity ranging from mild ketonuria to moderate ketoacidosis. The remarkable correspondence between the amount of amino acid added and the amount of MMA excreted confirms that urine MMA was mainly derived from diet protein. There was a temporal lag between intake of the toxic amino acids and the output of an equivalent excess of MMA in the urine. Since Stokke, *et al.*[3] estimated the half time of disappearance of MMA from plasma to be only 3 hours, the observed lag is not likely to be due to delayed renal clearance but may reflect slow equilibration of increased tissue levels of MMA or its precursors with plasma MMA.

MMA itself does not seem to be toxic. Peak plasma and urinary concentrations of MMA in R.P. did not coincide with clinical symptoms, and he has improved steadily in health and growth even though his urinary MMA has always been at least 50 times normal. Methylmalonyl-CoA, however, has been shown to inhibit pyruvate carboxylation and gluconeogenesis.[18] This

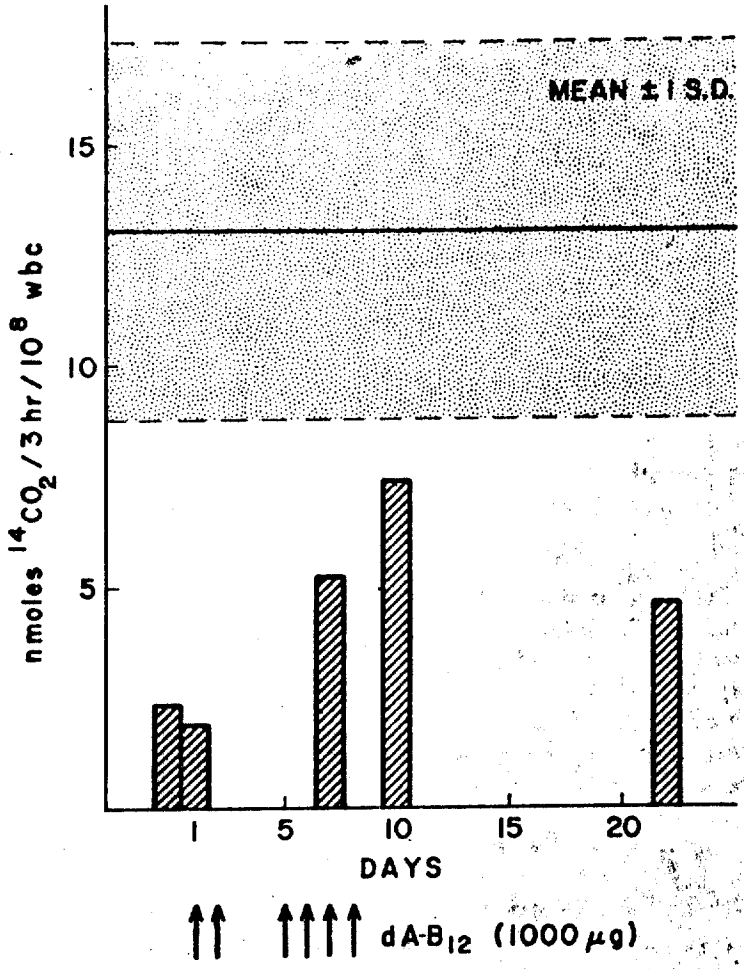

FIG. 5. Methylmalonate oxidation by leucocytes from R.P. while receiving injections of dA-B_{12}. The stippled area represents one standard deviation above and below the mean for oxidation of methyl-^{14}C-malonate (16.5 μmoles/ml) by normal leucocytes. The columns represent the activity of R.P.'s leucocytes in relation to daily injections of dA-B_{12}. His leucocytes showed defective oxidation of methylmalonate which was unchanged 24 hours after the first injection, but then increased to near normal levels. Enhanced activity was still apparent 2 weeks after the last injection of B_{12} was given.

suggested to Oberholzer, et al.[2] that the ketoacidosis in patients with methylmalonicaciduria might be a secondary manifestation of such enzyme inhibition. Other mechanisms are possible. Lindblad, *et al.*[7] suggested that accumulation of methylmalonyl-CoA sequesters all available intracellular CoA, thereby preventing acetyl-CoA production and decelerating the citric acid cycle. This mechanism seems unlikely since tissue concentrations of free CoA rose more than that of MMA in sheep made B_{12} deficient experimentally.[19] The acidosis could also be due to lactate accumulation. A minor pathway for the conversion of propionate to lactate has been found in human cells.[20] This pathway could become an important route for propionate catabolism if the normal major pathway to succinate were blocked, as it is in methylmalonicaciduria. Blood lac-

tate did rise in our patient when acidosis was induced by valine.

Acetone accounts for virtually all of the neutral urinary ketones in patients with ketosis secondary to diabetes mellitus, starvation, infection, or ingestion of ketogenic diets. In contrast, R.P. excreted considerable quantities of the four-carbon ketone, butanone and a six-carbon ketone, hexanone, during periods of spontaneous ketosis or after protein or amino acid-induced ketosis. This unique pattern of ketonuria, which has been observed previously[21] only in a patient now known to have propionyl-CoA carboxylase deficiency,[22] indicates that long chain ketones will be formed and excreted if the major pathway of propionate catabolism is blocked. These ketones could be formed by decarboxylation of propionate precursors. Thus, decarboxylation of α-methylacetoacetyl-CoA, a normal intermediate in the catabolism of isoleucine to propionate, yields butanone. The long chain ketones could also be formed by decarboxylation of fatty acids with an odd number of carbon atoms if more of these are synthesized from propionyl-CoA when the catabolism of this fatty acid primer is blocked. These fatty acids may also contribute to the metabolic acidosis.

Vitamin B_{12} Dependency

Five of the thirteen patients with congenital methylmalonicaciduria have responded to pharmacologic doses of B_{12}.[4,6,9,12] This B_{12} responsiveness (or dependence) has been noted *in vivo* and *in vitro*. *In vivo*, it has taken the form of a marked reduction in urinary MMA excretion under basal conditions or after an amino acid load. In R.P., daily intramuscular injections of CN-B_{12} produced a 70% reduction in MMA excretion. This response was seen only with more than 200 times the amount of B_{12} needed by normal individuals (> 200 μg daily compared to the daily physiologic requirement of < 1 μg). In R.P. the B_{12} dependency has clinical as well as biochemical significance. In the absence of B_{12} supplements, an oral valine load produced ketosis within 48 hours and all of the administered valine was excreted as MMA. An identical load of valine, however, given when the patient was receiving 1,000 μg of CN-B_{12} daily, produced no ketosis, and only one third of the valine load was excreted in the urine as MMA. Thus, B_{12} exerted a protective effect in the face of an otherwise toxic chemical stress.

Peripheral leukocytes and cultured fibroblasts have been used to study the B_{12} responsiveness *in vitro*. As reported earlier[23] and demonstrated in the present study, oxidation of labelled propionate or methylmalonate to $^{14}CO_2$ by peripheral leukocytes was distinctly enhanced when the patient was being treated with injections of CN-B_{12} or dA-B_{12}. Again, this response was observed only when B_{12} was given *in vivo*, not when the vitamin or its coenzyme was added to the incubation mixture. Since we have demonstrated recently that the primary defect in R.P.'s cells is their inability to synthesize dA-B_{12} from precursor cobalamins,[17,18] the failure of dA-B_{12} to correct methylmalonate metabolism completely *in vivo* or *in vitro* implies either that dA-B_{12} is poorly transported into cells or that this adenosylated compound is very unstable when injected.

Significant observations concerning B_{12} dependency have been reported by other investigators. Morrow, *et al.*[24] found that the dA-B_{12} content of the liver of one patient with methylmalonicaciduria was much reduced whereas three other patients had normal hepatic concentrations of this B_{12} coenzyme. Although the former patient did not receive B_{12} *in vivo* before his death, hepatic methylmalonyl-CoA mutase activity was increased to normal values by the *in vitro* addition of dA-B_{12}. Presumably, this patient had the B_{12} dependent variant of methylmalonicaciduria, whereas the other three patients had the B_{12} unresponsive form of the disease, which is almost certainly caused by a mutation in the gene for the mutase apoenzyme.

Mudd and his co-workers[25] found that the liver of a 7-week-old male infant who had

excess urinary MMA contained very reduced amounts of dA-B_{12}. Since this patient also had homocystinemia and cystathioninemia, it was suggested that he suffered from an inborn error of B_{12} metabolism which affected both B_{12} dependent reactions known in man, the isomerization of methylmalonyl-CoA to succinyl-CoA, and the remethylation of homocysteine to methionine, for which methyl-B_{12} is an essential coenzyme. Unlike this patient, R.P. had no abnormality in sulfur amino acid metabolism and must have a different defect of vitamin B_{12} metabolism affecting only dA-B_{12} and sparing methyl B_{12}.

Genetics

Methylmalonicaciduria is presumed to be congenital and inherited, but proof of this is lacking. Stillbirths and neonatal deaths have been reported in siblings of affected patients, but there is no documented recurrence of the disease in more than one child in a family. Since both males and females have been reported with the two variants of methylmalonicaciduria, autosomal recessive inheritance seems likely, but the demonstration of biochemical abnormalities in the presumed heterozygous parents of affected children has yet to be accomplished.

IMPLICATIONS

The diagnosis of methylmalonicaciduria should be considered in any infant with severe ketoacidosis, and in children with growth or developmental retardation. Its clinical phenotype is indistinguishable from that of other causes of infantile ketoacidosis. Therefore, the diagnosis depends on the detection of large amounts of MMA in the urine and on the exclusion of B_{12} deficiency (which can also cause growth retardation[6]). The diagnosis can be confirmed by demonstrating a block in methylmalonate oxidation by leucocytes, blood platelets, or cultured fibroblasts. Since patients with methylmalonicaciduria may exhibit hyperglycinemia, lactic acidosis, hyperammonemia, hypoglycemia, and transient granulocytopenia and thrombocytopenia, this disorder must be considered in the differential diagnosis of any of these findings.

Treatment of methylmalonicaciduria is based on dietary restriction of the toxic amino acids by a low protein diet. Such a diet led to remarkable improvement in R.P.'s course, even without B_{12} supplementation. All patients with methylmalonicaciduria should be given a therapeutic trial of large doses of vitamin B_{12}. This is the simplest way to identify those patients in this study with the B_{12} dependent form of the disease who will benefit both chemically and clinically from such vitamin supplements.

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Biochemical Functions and Interrelationships of Folic Acid and Vitamin B_{12}

F. M. HUENNEKENS

BIOCHEMISTRY is a rapidly growing and complex discipline, and it is understandably difficult to select those discoveries that have contributed most decisively to its present state of development. The recognition of vitamins as substances essential for the growth and well-being of living systems must be placed high on the list of fundamental advances in both biochemistry and nutrition. As recounted in the excellent text by Wagner and Folkers,[41] the story of vitamins began in the early 1900's with the empirical observations that certain diseases in man could be cured by appropriate additions to the diet. Subsequently, it was learned that many of the symptoms could be produced artificially by maintaining animals on synthetic diets and, further, that these disorders could be alleviated by supplementing the diets with extracts from natural sources such as liver and yeast. From these extracts, a number of vitamins were eventually isolated in pure form, their structures determined, and their availability assured by chemical synthesis. All of the presently known vitamins are relatively complex organic compounds, many of which contain heterocyclic rings as part of their structure.

Sir Frederick Hopkins, who founded and led the great school of biochemistry at Cambridge from 1914 to 1943, suggested that the effectiveness of vitamins at low levels must be due to the fact that they participate *catalytically* in metabolic processes. This hypothesis received further support in 1935 by Warburg's discovery that a TPNH*-oxidizing enzyme isolated from yeast contained a bound derivative of vitamin B_2 (riboflavin). Removal of this factor (later identified as FMN) inactivated the enzyme; addition of pure FMN fully restored the activity. FMN was thus established as the *coenzyme* for this TPNH-oxidizing enzyme and its participation in the actual catalytic process was demonstrated shortly thereafter. In 1941, Green proposed a generalized "vitamin-trace substance" theory[13] which stated that any substance required in the diet at "trace" levels must be part of an enzyme or a coenzyme. The 1940's and 1950's witnessed the discovery of all of the vitamins presently known and, at least in the case of the water-soluble, or B-, vitamins, Green's hypoth-

*The following abbreviations are used: DPN and TPN, di- and triphosphopyridine nucleotide; DPNH and TPNH, reduced DPN and TPN; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; P, inorganic phosphate; PPP, tripolyphosphate; CoA, coenzyme A; ADP and ATP, adenosine di- and triphosphate; CDP, dCDP and dCMP, cytidine diphosphate, deoxycytidine diphosphate and deoxycytidine monophosphate; C_1, one-carbon unit; FH_4, tetrahydrofolate; Lip (SH, SH) and Lip (S, S) dihydrolipoic and oxidized lipoic acid; FIG, formiminoglycine; FIGLU, formiminoglutamic acid.

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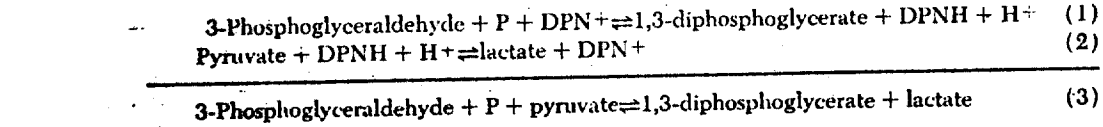
TABLE 1.—*Coenzymes as Carriers of Mobile Metabolic Groups*

Vitamin	Coenzyme	Mobile Group
Niacin	DPN, TPN	H^-
Pantothenic	CoA	Acyl
Lipoic	Lipoic	Acyl
Riboflavin	FAD, FMN	e^-
Pyridoxal	Pyridoxal P	Amino
Thiamine	Thiamine PP	Aldehyde
Folic	Tetrahydrofolic	Formyl, Formimino, Methenyl Methylene Methyl
Biotin	Biotin	CO_2
B_{12}	Adenosyl-B_{12}	H^- (?)
	Methyl-B_{12}	(?)

esis was confirmed. Two general rules were evident: (a) in tissues, the B-vitamins are converted to their coenzyme forms*; and (b) the coenzymes are bound to specific enzymes and participate in the catalytic processes. The fat-soluble vitamins, on the other hand, remain somewhat obscure with regard to both coenzyme form and function.

More precisely, each coenzyme is associated with a particular type of enzyme-catalyzed reaction (Table 1). For example, DPN, TPN, FAD and FMN are each components of dehydrogenases and oxidases; pyridoxal phosphate is involved with transaminases, racemases, decarboxylases and other enzymes metabolizing amino acids†; coenzyme A participates in reactions involving transformation of organic acids. This specificity, moreover, provides a valuable clue to the general function of coenzymes, namely to carry *mobile metabolic groups* during enzymic catalysis.[16] This point can be illustrated by considering the role of DPN in the linked oxido-reduction reactions of glycolysis:



3-Phosphoglyceraldehyde + P + DPN+ ⇌ 1,3-diphosphoglycerate + DPNH + H+ (1)
Pyruvate + DPNH + H+ ⇌ lactate + DPN+ (2)

3-Phosphoglyceraldehyde + P + pyruvate ⇌ 1,3-diphosphoglycerate + lactate (3)

In reaction (1), a pair of electrons (actually in the form of a hydride ion, H^-) is transferred from the donor (3-phosphoglyceraldehyde) to the acceptor (DPN); the concomitant esterification of inorganic phosphate (P) is important metabolically but not germane to the present discussion. In reaction (2), a hydride ion is returned from DPNH to pyruvate. The net reaction, shown in reaction (3), thus involves the transfer of a hydride ion from 3-phosphoglyceraldehyde to pyruvate. The coenzyme DPN acts catalytically as an intermediate carrier of the hydride ion, which is the "mobile metabolic group."

*Lipoic acid and possibly also biotin are apparent exceptions to this rule since they appear to function as coenzymes without being converted into more complex structures.

†An exception to this generalization is the fact that pyridoxal phosphate also serves as the coenzyme for glycogen phosphorylase (glycogen + P ⇌ glucose-1-P).

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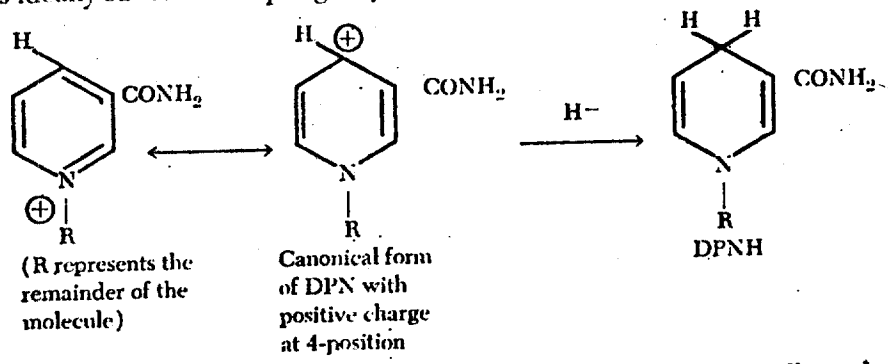
The relationship between donor and acceptor metabolite, mobile group and coenzyme, may be expressed by the following generalized equations:

$$\text{D-X} + \text{C} \rightleftharpoons \text{D} + \text{C-X} \qquad (4)$$
$$\text{C-X} + \text{A} \rightleftharpoons \text{C} + \text{A-X} \qquad (5)$$
$$\overline{\text{D-X} + \text{A} \rightleftharpoons \text{D} + \text{A-X}} \qquad (6)$$

The donor metabolite (D-X), which contains a potential mobile group (X), interacts with the coenzyme (C) to form (C-X), a complex between the coenzyme and the mobile group. It should be emphasized that (C-X) is a chemical compound, usually of sufficient stability to be synthesized chemically or enzymatically (via reaction 4 or reaction 5 in reverse). The acceptor metabolite (A) reacts with (C-X) and the mobile group is again transferred, yielding (A-X) and regenerating the coenzyme (C) for a repetition of the process. The net reaction (reaction 6) represents the transfer of a mobile group from donor to acceptor. One may ask, though, why the mobile group is not transferred *directly* from donor to acceptor? It may be more feasible thermodynamically for the reaction to be separated into two steps each with a small change in free energy or, alternatively, the interposition of a coenzyme may provide a means for regulating the transfer of the metabolic group.

In light of the above concept, the structural features of each vitamin become more meaningful since the mobile group must be attached to the vitamin portion of the coenzyme. Reactions (1) and (2), for example, would not be possible if the nicotinamide portion of DPN were not an aromatic heterocycle that is ideally suited to accepting a hydride ion at the 4-position:



(7)

Similarly, the other mobile groups listed in Table 1 are structurally and mechanistically compatible with their coenzymes. Conversely, the functional groups found on the protein portion of enzymes (carboxyl, α- and ϵ-amino, aliphatic and aromatic hydroxyl, thiol and imidazole) are apparently not suited for carrying these particular mobile groups.

Folic acid (1941) and vitamin B_{12} (1948) were among the last of the B-vitamins to be discovered and their biochemical functions, especially in the latter case, are still not entirely elucidated. This is due primarily to the fact that these two vitamins occur at low levels in tissues and therefore relatively few enzyme systems make use of their coenzyme forms. Folate- and B_{12}-dependent enzymes play key roles, however, in the synthesis of nucleic acids

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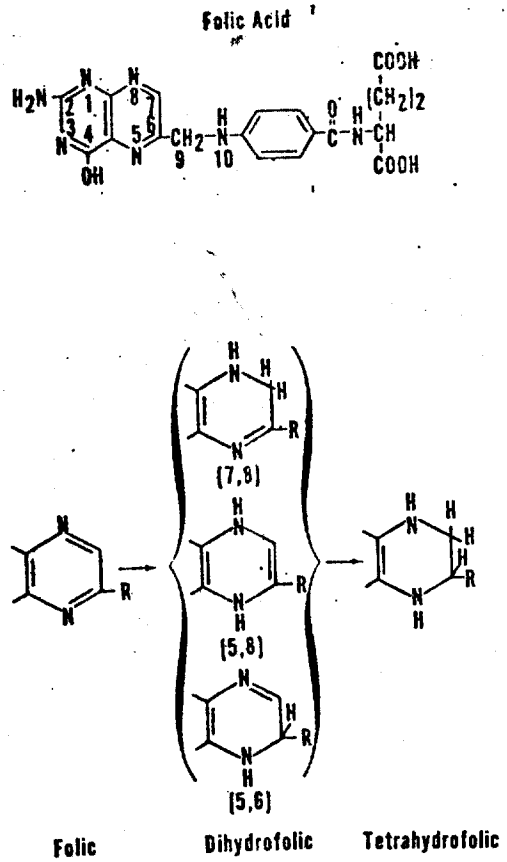


FIG. 1.—Structure of folic, dihydrofolic and tetrahydrofolic acid.

and proteins[19] and hence in the process of cell replication. The present paper is a brief review of the biochemical functions and interrelationships of folic acid and vitamin B_{12}, with emphasis upon the conversion of the vitamins to their coenzyme forms and the mechanisms by which the coenzymes carry mobile groups.

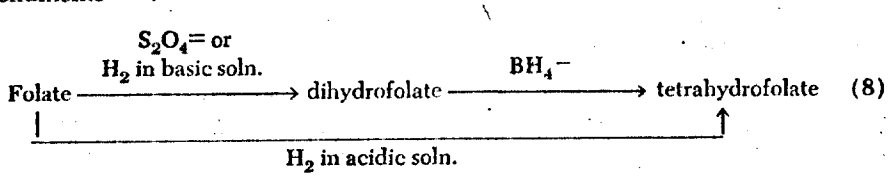
FOLATE COENZYMES

Conversion Of Folate To Tetrahydrofolate

In 1954, Blakley and Sakami independently reported that tetrahydrofolate (Fig. 1) was the coenzyme for the enzymatic reaction in which a one-carbon group is added to glycine to form serine. This significant finding initiated numerous investigations on the chemical and enzymatic reduction of folate to tetrahydrofolate. The chemical studies were especially useful, not only because they made dihydrofolate and tetrahydrofolate available in quantity, but also because they provided some insight into mechanisms for reduction of the pyrazine ring. Thus, when folate is treated with hydrosulfite* or is hydro-

*At elevated temperatures (about 70° C), hydrosulfite reduces folate to the tetrahydro level.

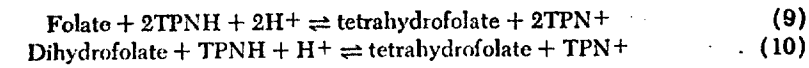
genated over platinum oxide in neutral or alkaline solution, dihydrofolate is the principal product. Tetrahydrofolate is produced when folate is hydrogenated in acid solution or when dihydrofolate is reduced with borohydride.* These reactions, outlined in equation (8), have been discussed elsewhere with respect to mechanisms[20,36]:



(8)

There has been considerable uncertainty over the exact structure of dihydrofolate, the intermediate in equation (8). Structures of three possible tautomeric forms† are illustrated in Figure 1. The 5,6-isomer was ruled out several years ago[29] by enzymatic evidence involving the stereoisomerism at C-6. It has been more difficult to decide between the 5,8- and 7,8-isomers, although recently the laboratories of Friedkin[31] and Blakley[14] have utilized tracer technics and nuclear magnetic resonance spectra to provide decisive evidence in favor of the latter structure.

The enzymatic reduction of folate closely resembles the non-enzymatic route. Enzyme preparations from various sources utilize reduced pyridine nucleotides (usually TPNH) to reduce either folate or dihydrofolate to the tetrahydro level‡ (cf. reactions (9) and (10)).



$$\text{Folate} + 2\text{TPNH} + 2\text{H}^+ \rightleftharpoons \text{tetrahydrofolate} + 2\text{TPN}^+ \quad (9)$$

$$\text{Dihydrofolate} + \text{TPNH} + \text{H}^+ \rightleftharpoons \text{tetrahydrofolate} + \text{TPN}^+ \quad (10)$$

In all instances in which highly purified preparations have been used (chicken liver, sheep liver, calf thymus, *Diplococcus pneumonii, Streptococcus faecalis, Lactobacillus leichmanii,* phage-infected *Escherichia coli,* and Ehrlich ascites tumor), a single enzyme (mol. wt. = 20,000 to 30,000) is found to catalyze both reactions or only reaction (10). Since the reduction of dihydrofolate is much faster than the reduction of folate, the enzyme is most properly called "dihydrofolate reductase." With dihydrofolate reductases from various sources, the Michaelis constants (K_m values) for dihydrofolate and TPNH are about equal, ca. 10^{-6} M, and the enzyme usually displays a curious double pH optimum with maxima at 4.5 and 7.5. Another interesting property of dihydrofolate reductases, discovered independently by Bertino[32] and Kaufman,[21,22] is the pronounced stimulation of the catalytic activity by K^+, *p*-chloromercuribenzoate or urea.

Of considerable importance to cancer chemotherapy is the fact that dihydrofolate reductases are strongly inhibited by the folic acid antagonists, Aminopterin, Amethopterin (Methotrexate), and Dichloroamethopterin.[18] The

*Borohydride will also reduce folate, but at a much slower rate than dihydrofolate.

†Kaufman[23] has suggested two additional structures (*o*- and *p*-quinonoid) for dihydrofolate.

‡Dihydrofolate is very probably an intermediate in reaction (9), but as yet no conditions have been found to demonstrate its accumulation.

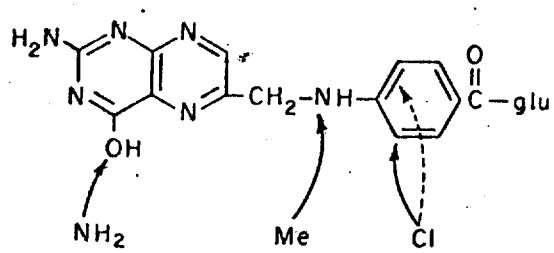


FIG. 2.—Structures of folic acid antagonists. Aminopterin = 4-amino folic acid; Amethopterin = 4-amino-10-methyl folic acid; Dichloroamethopterin = 4-amino-10-methyl-3',5'-dichloro folic acid. Parent structure is folic acid; glu = glutamic acid; Me = methyl.

structures of these compounds are shown in Figure 2. The inhibitor constant (K_I value) is approximately 10^{-9} M for each of these drugs. The inhibition appears to be stoichiometric, which permits the enzyme to be "titrated" by the antagonist. In leukemic leukocytes, dihydrofolate reductase is extremely sensitive to these drugs[18] and it is quite likely that this enzyme is the *only* protein in the cell that has an appreciable avidity for anti-folate compounds. Since dihydrofolate reductase is involved, moreover, as a component of the "thymidylate synthesis cycle," as discussed later, it is not surprising that folate antagonists repress leukocyte replication. In human leukemic patients, the acquired resistance to folate antagonists can be correlated to some degree with an increase in the amount of the target enzyme, dihydrofolate reductase.[18] This unfortunate effect appears to be due to a drug-stimulated "induction" of new enzyme rather than to a process of cell selection; the latter explanation is more likely to be applicable to the development of resistance in bacteria or cells in culture.

In *Clostridium sticklandii,* folate is reduced only to dihydrofolate by a folate reductase which utilizes for reducing power the Coenzyme A-dependent oxidation of pyruvate (reaction (11)), rather than TPNH or DPNH.

$$\text{Folate} + \text{pyruvate} + \text{CoA} \longrightarrow \text{dihydrofolate} + \text{acetyl CoA} + CO_2 \qquad (11)$$

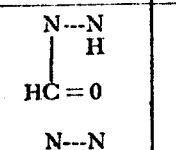
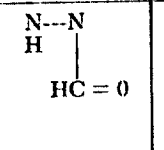
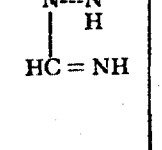
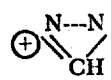
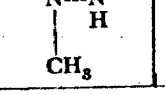
This enzyme is insensitive to folic acid antagonists in contrast to a conventional TPNH-dependent dihydrofolate reductase, which is also present in the organism. It has been shown recently[40] that the electron transfer sequence from pyruvate to folate in reaction (11) involves ferredoxin, a low molecular weight iron protein.

Functions Of Folate Coenzymes

The discovery of folic acid was followed shortly thereafter by the elucidation of its structure, as shown in Figure 1. As isolated from tissues, folic acid usually occurs as a polyglutamate, although most in vitro enzymatic studies have utilized the chemically-synthesized monoglutamate. Very little difference has been observed in enzyme systems between the monoglutamate and the polyglutamate forms of the folate coenzymes.

As described earlier, studies on the interconversion of serine and glycine revealed that tetrahydrofolic acid was the coenzyme form of folic acid.

TABLE 2.—C_1 *Groups and Their Sites of Attachment to Tetrahydrofolate*

Site of Attachment			C_1 Unit	Oxidation State
N^5	N^{10}	N^5 and N^{10}		
			formyl	formate
			formimino	formate
			methenyl	formate
			methylene	formaldehyde
			methyl	methanol

From a chemical viewpoint, reduction of the pyrazine ring permits the N-5 position of the folate molecule to enter into covalent linkages with one-carbon (C_1) groups. But it is not only the N-5 position to which the mobile groups become attached. The N-10 position, as well, carries C_1 units and, in some instances, the C_1 group can be bridged across both nitrogens, forming a 5-membered ring. Still another element of complexity is the fact that C_1 units, carried by tetrahydrofolate, can exist at three different oxidation states: formate, formaldehyde and methanol. Such multiplicities in the nature of the mobile group or its site of attachment to the coenzyme are not ordinarily encountered with the other examples of coenzymes and mobile groups listed in Table 1. Fortunately, all of the possible permutations and combinations between C_1 groups and sites of attachment are not encountered in nature. As shown in Table 2, the known complexes include: 5-formyl, 10-formyl, 5-formimino, 5,10-methenyl, 5,10-methylene and 5-methyl tetrahydrofolate. The 5-membered ring in 5,10-methenyl tetrahydrofolate has two resonance structures depending on the position of the double bond and the corresponding positive charge on one of the nitrogen atoms. At the formaldehyde level, it is not surprising to find only one possibility, namely the 5,10-methylene derivative of the tetrahydrofolate. If a formaldehyde group were attached as $-CH_2OH$ to only one of the nitrogen atoms, the resulting N-hydroxymethyl compound would be an easily dissociable equilibrium adduct, and would probably not be suitable for metabolic reactions. It is possible, of course, that the other potential compounds missing from Table 2 actually exist, but as yet no evidence has been obtained for their participation in enzymatic reactions.

The diagram in Figure 3 summarizes the reactions in which transfer of

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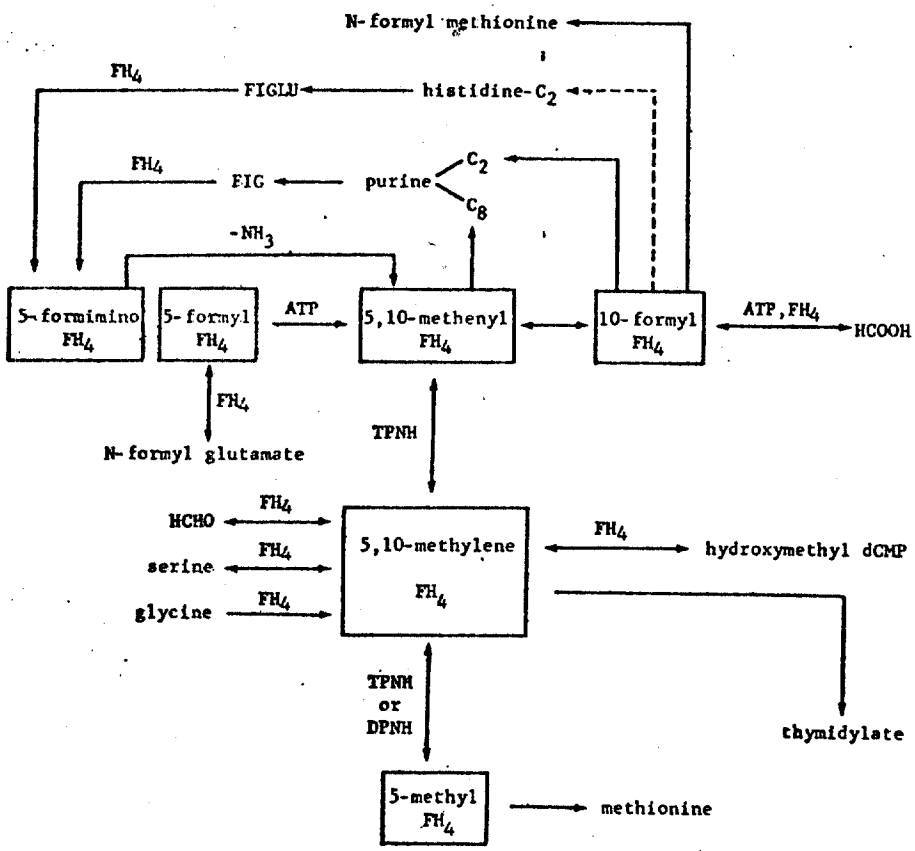

FIG. 3.—Metabolic reactions involving folate coenzymes.

C_1 units between various donor and acceptor metabolites is mediated by folate coenzymes.[12,17,19,34] The six known C_1-tetrahydrofolate intermediates (see Table 2) are enclosed in boxes to emphasize their function as transient repositories of the mobile groups. The formation and breakdown of these complexes coupled with the release or acceptance of C_1 units by various metabolites are indicated by reaction arrows; double-headed arrows indicate reversible reactions.

In discussing these interdigitated pathways, it is convenient to begin with the reaction at the top right, whereby free formate is brought into the "active C_1" pool via reaction (12):

$$\text{Formate} + \text{tetrahydrofolate} + \text{ATP} \overset{Mg^{++},\,K^{+}}{\rightleftharpoons} \text{10-formyl tetrahydrofolate} + \text{ADP} + \text{P} \quad (12)$$

This reaction, which is catalyzed by formate-activating enzyme (tetrahydrofolate formylase, formyl tetrahydrofolate synthetase), is probably only of minor importance in mammalian tissues. In certain bacteria (e.g., *Clostridium cylindrosporum* from which it has been obtained in crystalline form), the enzyme may comprise as much as several per cent of the soluble protein. Rabinowitz[34a] has suggested that in these bacteria 10-formyl tetrahydrofolate

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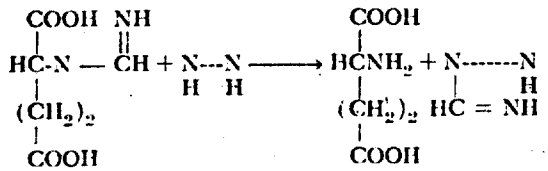
(arising from the breakdown of purines via formiminoglycine, as described below) may be used to synthesize ATP by a reversal of reaction (12). As with many other "activation" reactions in which the scission of ATP is coupled to the synthesis of a covalent bond, the mechanism of reaction (12) is still not entirely clear.

Two of the forms of "active formate," i.e., the 10-formyl and the 5,10-methenyl derivatives of tetrahydrofolate, are in equilibrium with each other and can be interconverted via the enzyme, cyclohydrolase, or non-enzymatically merely by changing the pH:

$$[\text{5,10-Methenyl tetrahydrofolate}]^+ + H_2O \rightleftharpoons \text{10-formyl tetrahydrofolate} + H^+ \quad (13)$$

As shown by the extensive studies of Buchanan[9a] and G. R. Greenberg,[13a] 10-formyl tetrahydrofolate serves as the immediate precursor of carbon-2 of inosinic acid, the purine nucleotide arising by de novo synthesis from small molecular weight precursors. Conversely, carbon-8 of the purine ring is derived from 5,10-methenyl tetrahydrofolate. By analogy with the above information, it is probable that 10-formyl tetrahydrofolate, rather than the 5,10-methenyl derivative, is the immediate precursor of carbon-2 of the histidine ring; because of the uncertainty, however, this reaction is shown by a dashed line on the diagram. Recently, Adams and Capecchi[1a] and Marcker[28] have demonstrated that 10-formyl tetrahydrofolate (or possibly the methenyl derivative) is the precursor of N-formyl methionyl-sRNA; blocking the amino group thereby renders this amino acid ideally suited for the initiation of polypeptide chains.

In degradation of the purine and histidine rings, the potential C_1 units do not emerge as formate, as would be expected if breakdown were merely the reversal of synthesis. Instead, the carbon and the adjacent nitrogen atoms emerge as a formimino group $\overset{NH}{\underset{HC-}{\|}}$. Thus, histidine and purines are degraded, respectively, to formiminoglutamic acid (FIGLU) and formiminoglycine (FIG), and both of these intermediates react with tetrahydrofolate* to yield 5-formimino tetrahydrofolate (see reaction (14) for the reaction of FIGLU).



(14)

The latter compound subsequently loses ammonia via a cyclization reaction (15) to yield 5,10-methenyl tetrahydrofolate. By this sequence of reactions, the potential C_1 units in the histidine and purine rings are returned to the C_1 pool.

$$\text{5-Formimino tetrahydrofolate} + 2H^+ \longrightarrow [\text{5,10-methenyl tetrahydrofolate}]^+ + NH_4^+ \quad (15)$$

*The symbol $\underset{H}{N}$- - -$\underset{H}{N}$ is used to represent the N-5 and N-10 positions of tetrahydrofolate.

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5-Formyl tetrahydrofolate, still another form of "active formate," has a somewhat uncertain function in intermediary metabolism. Isolated from natural sources shortly after the discovery of folic acid, it was assumed with some justification to be an intermediate in various enzymatic reactions, such as those responsible for the incorporation of C_1 units into the 2- and 8-positions of the purine ring. However, except for the interaction of N-formyl glutamate with tetrahydrofolate (catalyzed probably by the enzyme responsible for reaction (14)), no other reactions are known which either produce or utilize 5-formyl tetrahydrofolate. This compound may represent a storage form of folic acid, since it can be brought into the mainstream of C_1 metabolism via an ATP-dependent isomerization reaction which yields 5,10-methenyl tetrahydrofolate:

$$\text{5-Formyl tetrahydrofolate} + \text{ATP} + \text{H}^+ \longrightarrow [\text{5,10-methenyl tetrahydrofolate}] + \text{ADP} + \text{P} \qquad (16)$$

At the next lower oxidation state of the C_1 unit, the only form of "active formaldehyde" encountered is 5,10-methylene tetrahydrofolate. This intermediate can be generated readily by the interaction of formaldehyde with tetrahydrofolate, although this reaction is probably of little importance in

$$\text{HCHO} + \text{tetrahydrofolate} \rightleftharpoons \text{5,10-methylene tetrahydrofolate} + 2\text{H}_2\text{O} \qquad (17)$$

mammalian or bacterial systems. Of much greater importance is the synthesis of 5,10-methylene tetrahydrofolate from the β-carbon of serine via reaction (18) catalyzed by serine hydroxymethylase:

$$\text{Serine} + \text{tetrahydrofolate} \overset{\text{Pyridoxal P}}{\rightleftharpoons} \text{glycine} + \text{5,10-methylene tetrahydrofolate} \qquad (18)$$

There is also a similar reaction, although less widely distributed in nature, in which glycine interacts with tetrahydrofolate* to form "active formaldehyde":

$$\text{Glycine} + \text{tetrahydrofolate} + \text{DPN}^+ \longrightarrow \text{CO}_2 + \text{NH}_4^+ + \text{5,10-methylene tetrahydrofolate} + \text{DPNH} \qquad (19)$$

Recent work by Sagers[26] and by our laboratory[2] has shown that the complex enzyme system from *Peptococcus glycinophilus* responsible for reaction (19) consists of four proteins: a pyridoxal-containing protein, a flavoprotein, a low molecular weight thioredoxin-like protein, and an unidentified protein.

Reactions which utilize 5,10-methylene tetrahydrofolate, in addition to reaction (18) in reverse, include the interaction of the coenzyme with deoxycytidylate (dCMP) to form 5-hydroxymethyl dCMP† and a more complex reaction with deoxyuridylate leading to the formation of thymidylate. In the first reaction, the methylene group is probably converted transiently to

---

*In rat liver, the α-carbon of glycine is converted to "active formate" by the following sequence of reactions: glycine $\longrightarrow$ glyoxylate $\xrightarrow{\text{glutamate}}$ CO_2 + N-formyl glutamate $\xrightarrow{FH_4}$ 5-formyl FH_4.

†The enzyme responsible for this reaction occurs in *Escherichia coli* after infection by the T-even bacteriophages.

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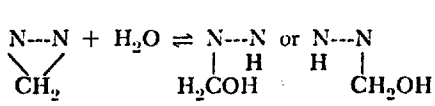
hydroxymethyl (reaction (20)) and the latter is transferred from tetrahydrofolate to the acceptor. In thymidylate synthesis, the C_1 group is simultaneously transferred and reduced to methyl level by a mechanism which will be discussed below.



(20)

At the lowest oxidation state of the C_1 unit, only the 5-methyl derivative of tetrahydrofolate occurs. This compound is the direct precursor of the methyl group of methionine via reaction (21) catalyzed by methionine synthetase.

5-Methyl tetrahydrofolate + homocysteine ⟶ methionine + tetrahydrofolate (21)

5-Methyl tetrahydrofolate (originally called "Prefolic A") was first isolated from natural sources by Keresztesy and Donaldson. Later, the laboratories of Buchanan and Sakami demonstrated that Prefolic A was, in fact, 5-methyl tetrahydrofolate. Although reaction (21) appears to involve a straightforward transfer of the methyl group from methyl tetrahydrofolate to homocysteine, some indication of the complexity is shown by the fact that a coenzyme form of B_{12} is required. The mechanism of the methionine synthetase reaction will be discussed subsequently.

The other example of methyl group synthesis mediated by folate coenzymes is the conversion of deoxyuridylate to thymidylate. This reaction is extremely important in cell replication since thymidylate, in the form of its triphosphate, is usually the rate-limiting nucleotide in DNA synthesis. The methyl group of thymidylate does not arise from 5-methyl tetrahydrofolate, as might be surmised by analogy with methionine synthesis, but rather from 5,10-methylene tetrahydrofolate. The overall reaction for the synthesis of thymidylate (reaction (22) was first established by D. M. Greenberg, Friedkin and Blakley. The C_1 unit is not only transferred from tetrahydrofolate to the pyrimidine, but it is simultaneously reduced to the methyl level. 5-Hydroxymethyl deoxyuridylate does not appear as an intermediate in this reaction and there is no evidence for involvement of a B_{12} coenzyme. Reduction of the C_1 unit is carried out at the expense of tetrahydrofolate being oxidized to dihydrofolate.*

5,10-Methylene tetrahydrofolate + deoxyuridylate ⟶ dihydrofolate + thymidylate (22)

The continuous synthesis of thymidylate requires that reaction (22), catalyzed by thymidylate synthetase, be coupled to the regeneration of 5,10-methylene tetrahydrofolate. The latter is accomplished by reconverting dihydrofolate to tetrahydrofolate via dihydrofolate reductase (reaction (10)), followed by addition of the C_1 unit from a metabolite such as serine (reaction (18)). It is interesting to note that this multi-step, cyclic process for the biosynthesis of thymidylate involves the target sites for two important chemotherapeutic agents: thymidylate synthetase is extremely sensitive to fluoro-

*In the hydroxylation of phenylalanine to tyrosine, tetrahydropteridines including tetrahydrofolate are also oxidized to the dihydro stage during their function as coenzymes.

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pyrimidines while dihydrofolate reductase is inhibited by folic acid antagonists.

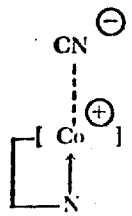
Referring back to Figure 3, it can be seen that C_1 units at the three oxidation states are interconvertible by means of the pyridine nucleotide-dependent enzymes: 5,10-methylene tetrahydrofolic dehydrogenase and 5,10-methylene tetrahydrofolic reductase. These enzyme systems bring about the oxido-reduction of C_1 units while the latter are attached to tetrahydrofolate. The connected pathways in Figure 3 thus demonstrate the facile interconversion of C_1 units in formate, purines, histidine, N-formyglutamate, formaldehyde, serine, glycine, hydroxymethyl deoxycytidylate, methionine, and thymidylate. Known enzymatic reactions now account fully for the early nutritional and tracer studies which pointed toward "C_1 pools" in equilibrium with the above metabolites.

IN SUMMARY, the conversion of folate (the vitamin) to tetrahydrofolate (the coenzyme) via the intermediate, dihydrofolate, may be carried out chemically or enzymatically. The enzyme responsible for this process, dihydrofolate reductase, has been isolated from several sources and is a well-characterized protein. The role of tetrahydrofolate as a carrier of C_1 groups at three different oxidation states is now understood and the structures of six C_1-tetrahydrofolate complexes are established. A series of enzymes ("pteroproteins"), which in some instances have been highly purified, catalyze the formation and breakdown of these C_1-tetrahydrofolate complexes as part of the biosynthesis and degradation of certain metabolites.[19] The precise mechanisms of these interesting reactions, for the most part, still remain to be elucidated.

B_{12} Coenzymes*

Conversion Of Vitamin B_{12} To Its Coenzyme Forms

The isolation of vitamin B_{12} as the cyanide complex in 1948 was followed shortly thereafter by the elucidation of its structure as shown in Figure 4. The "active center" of the molecule is the cobalt atom which is bonded to four pyrrole nitrogens. The pyrroles, in turn, comprise a nearly planar corrin ring which is similar to the biologically important porphyrins, except that one of the methine bridges linking the pyrroles is missing. In addition, the cobalt forms two other linkages with groups above and below the plane of the corrin ring. The cyanide group may be considered to be "above" the plane while the parent base (most commonly 5,6-dimethylbenzimidazole) is "below" the plane. The structure of cyanocobalamin can be written symbolically in order to emphasize the following salient features:



(a) the cobalt atom is trivalent and enclosed within a corrin ring ([ ]); (b)

*Several comprehensive reviews[6,10,33] on the chemistry and enzymology of vitamin B_{12} have appeared recently. The nomenclature of vitamin B_{12} and its derivatives has been discussed by Beck[4] and Bonnet.[9] The most commonly used form of vitamin B_{12}, in

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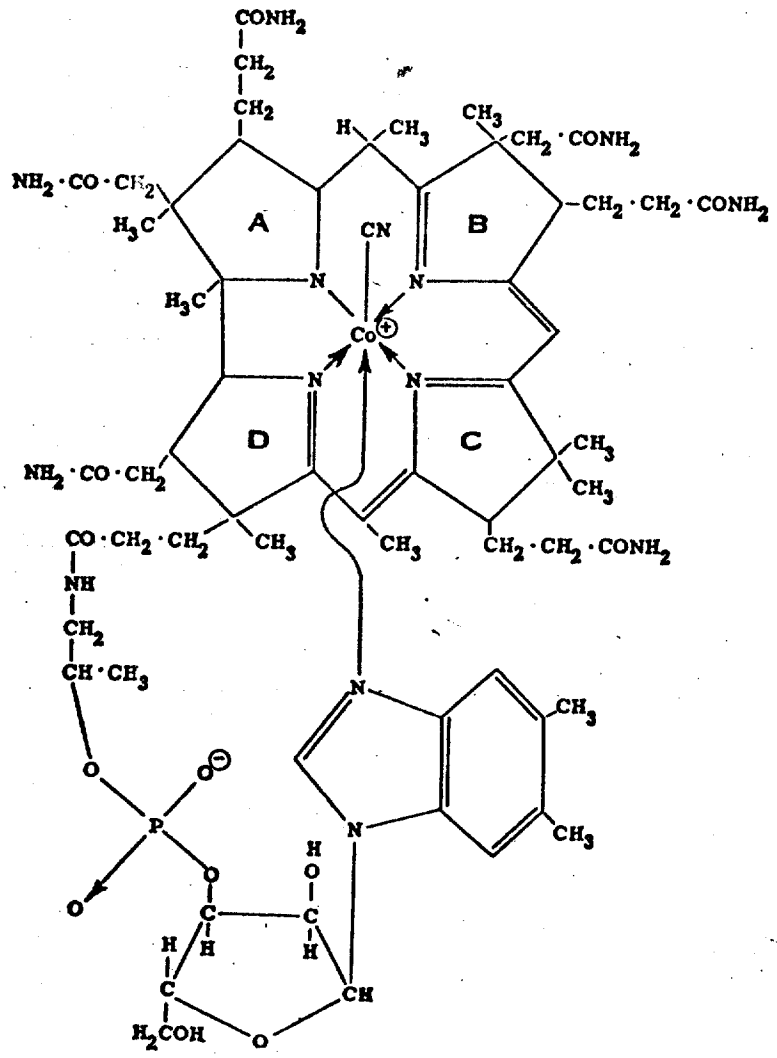

FIG. 4.—Structure of vitamin B_{12} (cyanocobalamin).

the cyanide group is attached ionically (---); (c) a nitrogen atom (N) of the parent base is linked to the cobalt by a coordinate bond (⟶); and (d) the parent base is joined by an extended structure (|—) to one of the pyrroles. This last structural feature may be important for mechanistic purposes since it would permit the parent base to swing away from the lower side without being irretrievably lost.

An understanding of the function of B_{12} in enzymatic systems was initiated with the isolation by Barker's group of adenosyl-B_{12}, a coenzyme form of the vitamin.[3] Adenosyl-B_{12} exists in relatively large quantities in certain bac-

which dimethylbenzimidazole is the parent base, is named "5,6-dimethylbenzimidazolylcyanocobamide" or "cyanocobalamin." The corresponding coenzyme, in which the CN-group is replaced by adenosine, is "dimethylbenzimidazole cobamide coenzyme" (abbreviated "DBC") or "adenosyl-B_{12}."

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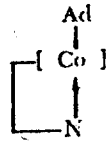
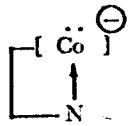
teria, notably *Clostridium tetanomorphum* or *Propionibacterium shermanii*. Degradation studies and x-ray analyses revealed that carbon-5 of the adenosyl group (Ad), replacing the cyanide above the plane of the ring, is linked *covalently* to the central cobalt atom:

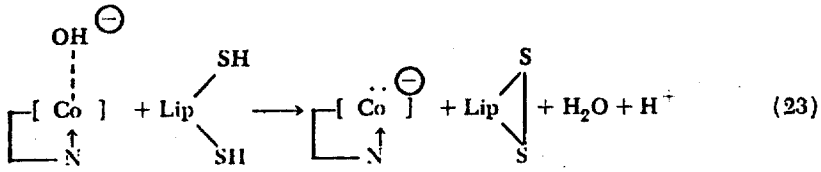


Barker, Weissbach and Brady first described an enzyme system responsible for the conversion of the vitamin to its coenzyme form. Extracts of the above bacteria, when supplemented with ATP, Mg^{++}, DPNH and FAD, were able to convert B_{12} or B_{12a}* to adenosyl-B_{12}. An important clue to the mechanism of this reaction was provided from studies on the chemical synthesis of adenosyl-B_{12}. It was shown by Smith and Johnson and by Müller and Müller that vitamin B_{12a} undergoes a two-step reduction when treated with borohydride: the first product is B_{12r} (in which the cobalt atom is in the +2 oxidation state) and the second product is B_{12s} (Co = +1). The latter compound, having a pair of electrons above the plane of the ring (see below), is a powerful nucleophile† which interacts readily with methyl



iodide or adenosine-5′-tosylate to yield methyl-B_{12} and adenosyl-B_{12}, respectively. These transformations, which are detailed in Figure 5, provide a convenient chemical route for the synthesis of alkyl derivatives of B_{12}.‡

The enzymatic synthesis of adenosyl-B_{12} in *Clostridium tetanomorphum* follows essentially the same sequence, i.e., reduction of B_{12} followed by adenosylation.[38] A B_{12a} reductase uses DPNH or dihydrolipoic acid for the reduction§ of B_{12a} to B_{12s} (reaction (23)) and a separate protein, called "the adenosylating enzyme,"[39] utilizes ATP as the source of the adenosyl group (reaction(24)). These reactions are also shown in Figure 5.



(23)

*Vitamin B_{12a}, which is identical to B_{12} except that CN^- is replaced by OH^-, is more reactive both in chemical and enzymatic systems and, for this reason, has been used in most investigations.

†B_{12s} may be considered as Co^{+1} with an attached proton or as Co^{+3} with an attached hydride ion (hydridocobalamin).

‡These compounds can be differentiated qualitatively by their colors: B_{12a} is pink; B_{12r}, orange; B_{12s}, grey-green; and methyl-B_{12} or adenosyl-B_{12}, red.

§In crude extracts of the bacteria but not with the partially purified enzyme, DPNH plus FAD can replace dihydrolipoic acid.

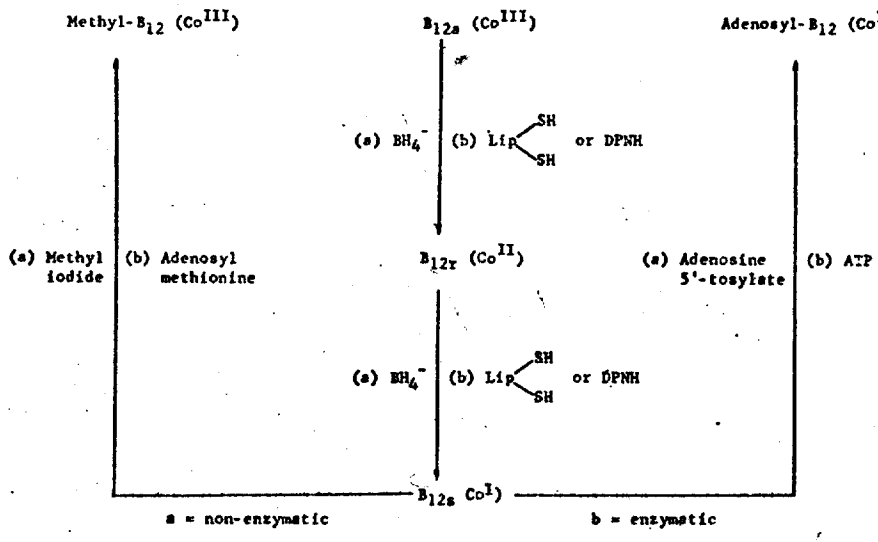
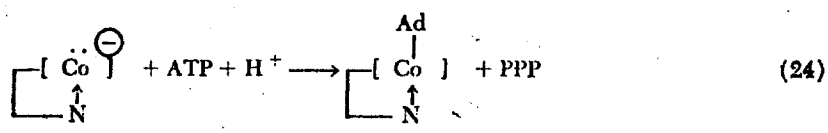


FIG. 5.—Chemical and enzymatic synthesis of B_{12} coenzymes.



(24)

Recent work from our laboratory[35,42] has provided further information about the mechanism of reaction (23). When dithiols are allowed to react *nonenzymatically* with B_{12s}, the sequence of events is shown in Figure 6. Initially, a thiol-B_{12} complex is formed (Step 1), but this is rapidly decomposed by the action of the neighboring, free thiol group: cleavage of the S-cobalt bond results in B_{12s} and the disulfide. B_{12s} will react with B_{12r} (present in excess in order to force the equilibrium of Step 1 to the right) to form B_{12r} or, if an acceptor such as methyl iodide (MeI) is present, it will react preferentially to form methyl-B_{12}. We believe that the enzymatic pathway utilizes the same general mechanism (Fig. 7), except that the dithiol grouping is situated on a thioredoxin-like protein. Recycling of this carrier protein to the reduced stage is carried out enzymatically by a DPNH-dependent flavoprotein (fp) or non-enzymatically by dihydrolipoic acid. Unlike free B_{12s}, which would be very labile to air-oxidation, the thioprotein-B_{12} complex would provide a means for generating B_{12s} as needed by the ATP-dependent adenosylating enzyme.

Function Of B_{12} Coenzymes

As in the previous discussion of folate coenzymes; it is useful to examine B_{12}-dependent reactions from the viewpoint of the mobile groups involved and the linkage of these groups to the coenzyme.

Concomitantly with the discovery of adenosyl-B_{12}, Barker and his colleagues demonstrated that this compound is the coenzyme for glutamate mutase, the enzyme responsible for the isomerization of L-glutamate to β-methylaspartate (reaction 1, Fig. 8). Similarly, the isomerization of succinyl CoA

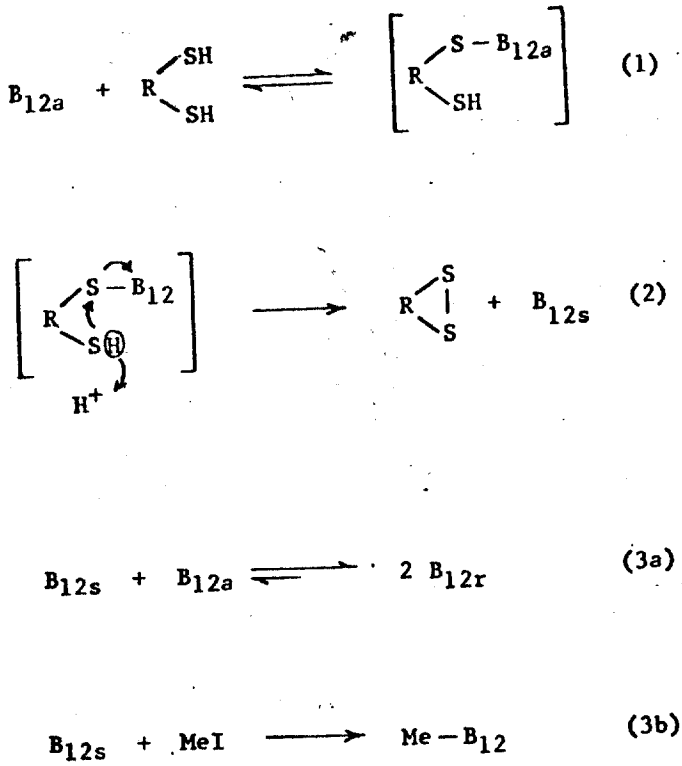


FIG. 6.—Non-enzymic reduction of B_{12a} with thiols.

to methylmalonyl CoA has been shown by Ochoa,[27a] and by Lynen and Stadtman[10a] to require adenosyl-B_{12} as a coenzyme (reaction 2). A third adenosyl-B_{12} system, studied by Abeles,[1] is an internal oxido-reduction reaction whereby propionaldehyde is converted to 1,2-propanediol (reaction 3). Although these reactions have been studied in detail with purified enzymes, relatively little is yet known about their mechanisms. In all three reactions, there is no net gain or loss of carbon or hydrogen atoms from the substrate. Tracer experiments support, but do not prove, the possibility of a hydride ion mechanism. The hydride ion, if it is the mobile group, would have to be detached from the substrate, sequestered transiently by the coenzyme,* and finally returned to the modified (isomerized) substrate. If the adenosyl group remains fixed to the cobalt atom during operation of the coenzyme, the hydride ion might be attached at the coordination site below the plane. That is, if the parent base were to swing away, this would permit replacement by H^-. This type of mechanism, admittedly speculative, for the participation of the adenosyl-B_{12} in isomerization reactions is shown in Figure 9.

Adenosyl-B_{12} is also involved in the pyridine nucleotide-dependent reduction

*Support for this hypothesis comes from recent experiments in Abeles' laboratory[11] in which tritium from propanediol is transferrred to the coenzyme (possibly to C-5 of the adenosyl group) and then back to the product, propionaldehyde.

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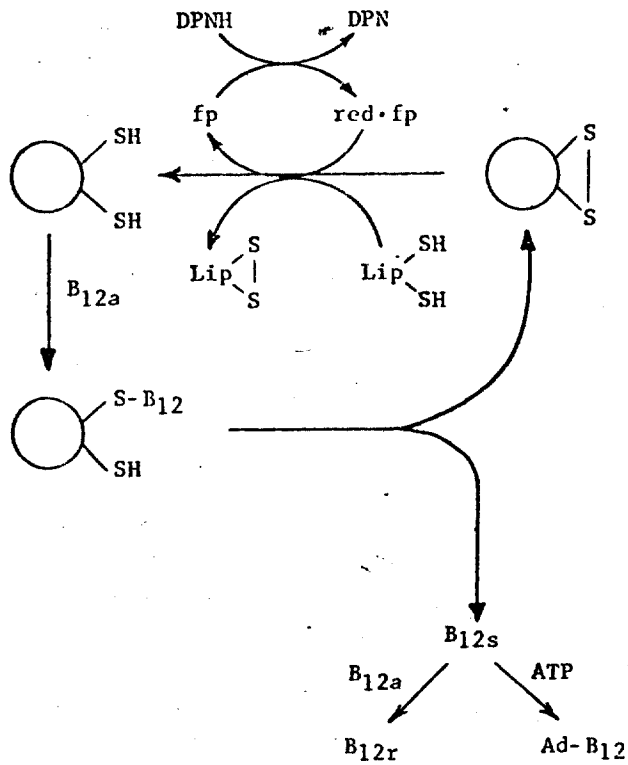
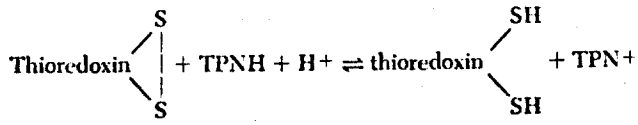


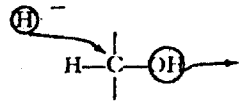
FIG. 7.—Enzymatic reduction of B_{12a}.

of ribonucleotides to deoxyribonucleotides; this reaction may have considerable significance in the direct conversion of RNA nucleotides to their DNA counterparts. Reichard has isolated, as part of a cytidine diphosphate reductase system from *E. coli*, thioredoxin reductase,[30] a flavoprotein (molecular weight = 60,000) that utilizes TPNH to reduce a disulfide bridge on thioredoxin[27] (molecular weight = 12,000):



(25)

Reduced thioredoxin probably supplies a hydride ion for reduction at C-2 of the sugar, but it is not yet clear how the OH is removed:



The *E. coli* system does not appear to contain or to require any B_{12} coenzyme,[15] in contrast to a similar system (TPNH- and thioredoxin-dependent) from *Lactobacillus leichmanii*, studied by Blakley[7] and by Beck and Reichard.[7]

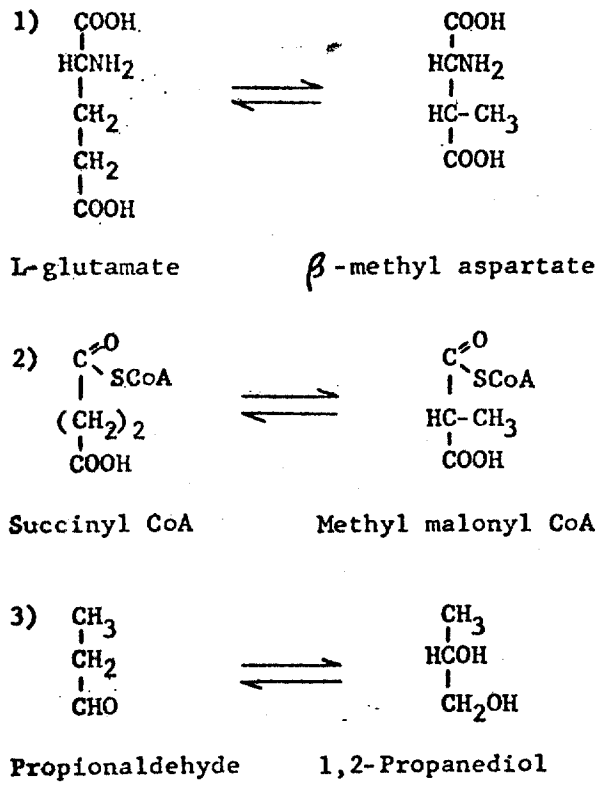
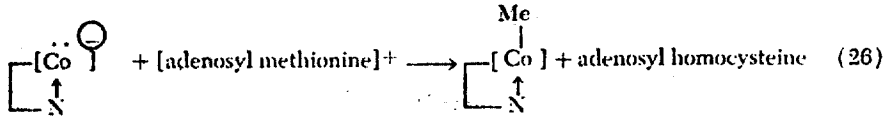


FIG. 8.—Isomerization reactions involving B_{12} coenzymes.

that has an absolute requirement for adenosyl-B_{12}. The latter system is specific for nucleoside *tri*phosphates.

As stated earlier, methyl-B_{12} has been shown to be involved in the synthesis of methionine,[37] according to reaction (21). In this instance, we have postulated the mechanism[24,25] shown in Figure 10 to account for the role of the B_{12} coenzyme in a methyl transfer reaction. The similarity of this mechanism to the previous one (Fig. 9) is apparent. The parent base is protonated (perhaps by a functional group on the enzyme itself) and swings away from the coenzyme in order to make room for 5-methyl tetrahydrofolate. The unshared electron pair on the N-5 of tetrahydrofolate would be attracted toward the electropositive cobalt atom and the resulting complex would facilitate the departure of the methyl group as a carbonium ion.

The biosynthesis of methyl-B_{12} is believed to follow a pathway similar to that outlined in reactions (23) and (24) for adenosyl-B_{12}. That is, B_{12a} is first reduced to B_{12s} and the latter reacts with adenosyl methionine:



Adenosyl methionine, along with DPNH and FAD, have been shown by several laboratories to be required cofactors in methionine synthesis. We

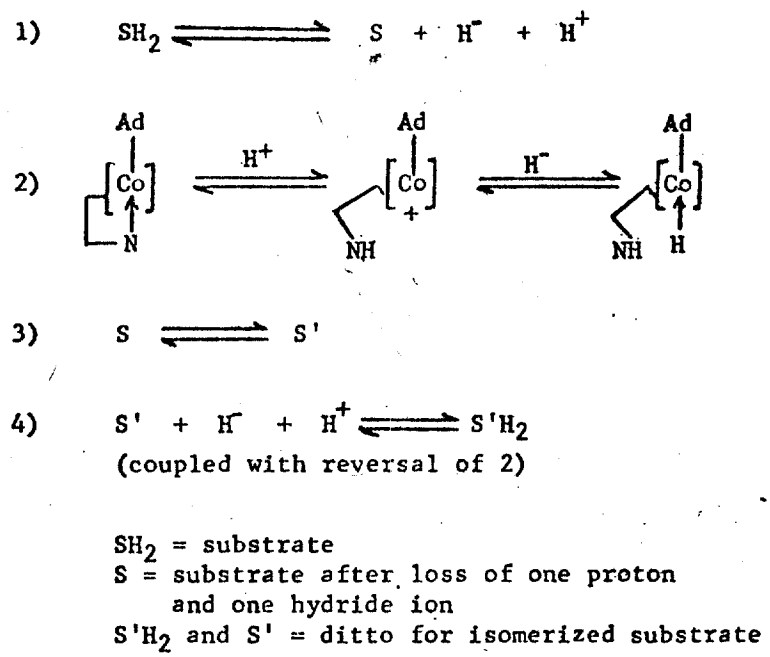


FIG. 9.—Hypothetical mechanism for role of adenosyl-B_{12} in isomerization reactions.

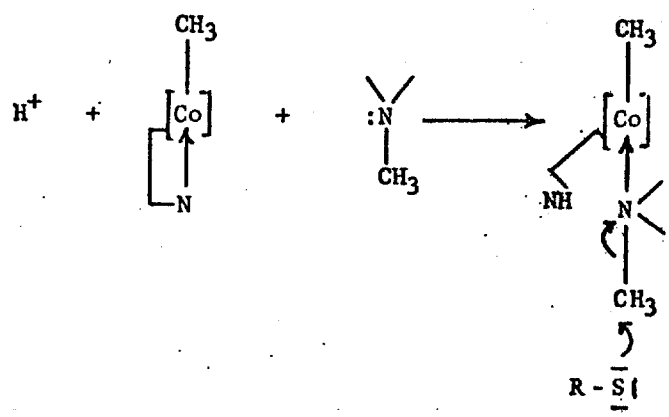


FIG. 10.—Hypothetical mechanism for role of methyl-B_{12} in methionine synthesis.

believe, however, that these components, along with accessory enzymes, are necessary only for the continued resynthesis of the somewhat labile methyl-B_{12}.

As shown recently by T. C. Stadtman[8] and by Wolfe,[43] methyl-B_{12} may also be involved in the formation of methane in bacteria such as *Methanosarcina barkeri*. If so, one would envision a reductive cleavage of methyl-B_{12} in order to yield B_{12s} and CH_4. Similarly, the observations by Wood[27b] and by E. R. Stadtman[33a] that carboxymethyl-B_{12} can serve as the source for acetate in certain bacteria suggests the possibility of a widespread alkyl-B_{12} reductase.

IN SUMMARY, two B_{12} coenzymes (adenosyl-B_{12} and methyl-B_{12}) are now readily accessible via chemical and enzymatic syntheses and their structures have been established. The

participation of these coenzymes in several enzymatic reactions has been demonstrated and, in some cases, the enzymes have been purified. What remains to be done, though, is to understand the *mechanism* of these reactions and particularly the role of each coenzyme as a carrier of the yet-unidentified mobile groups. Intrinsic factor, which binds B_{12} strongly, may be a useful tool for studying enzymes with tightly bound B_{12} coenzymes much in the same manner that avidin has been employed with biotin-containing enzymes.

Conclusion

Our present understanding of the biochemical function of folate and B_{12} coenzymes, although still incomplete, nevertheless provides a starting point for approaching certain clinical situations. Deficiencies of folic acid and vitamin B_{12}, whether produced accidentally in man or deliberately in experimental animals, birds or microorganisms, produce well defined and physiological manifestations. The paramount importance of these vitamins and their synergism in the production of blood cells has been stressed repeatedly by nutritionists and clinicians. Unfortunately, most biochemical studies to date have been concerned with the function of folate and B_{12} coenzymes in tissues other than blood cells.

Ideally, one would like to correlate each cellular lesion, resulting from a vitamin deficiency, with a specific biochemical event controlled by a coenzyme form of the vitamin. In the folate area, most of the pertinent enzymatic reactions have probably been discovered, but one is less sure about the B_{12} area. The reactions known to be mediated by B_{12} coenzymes would hardly appear to account for the far-reaching effects observed in B_{12}-deficiency. Additional B_{12}-dependent reactions very probably remain to be discovered, especially in nerve tissue where the effects of B_{12} deficiency are striking. At the present time, the involvement of a B_{12} coenzyme in deoxyribonucleotide synthesis, and possibly in methionine synthesis, appears to be the most promising lead for correlating biochemical events with physiological function. The interplay of B_{12} and folate coenzymes, especially in the production of megaloblastic cells, also provides a fruitful area for collaboration between biochemists and clinicians.

ACKNOWLEDGMENT

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Clinical Trial of the Effect of Vitamin B_{12} in Elderly Subjects with Low Serum B_{12} Levels

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Summary: A clinical trial of the effect of vitamin B_{12} therapy was conducted in 39 elderly subjects who had been found, in a community screening survey, to have low levels of serum B_{12} without a macrocytic anaemia or neuropathy. The study produced no evidence which suggests that in such subjects B_{12} is superior to placebo in effecting an improvement in psychiatric state or general well-being. There was a clear tendency for all the subjects to show an improvement during the trial, but this probably represents the therapeutic effect of involvement in a research exercise of this kind.

Introduction

Pernicious anaemia may be accompanied by psychiatric disturbance and in some instances the presenting illness may be psychiatric in nature, the anaemia coming to light only in the course of investigation (Langdon, 1905; McAlpine, 1929; Holmes, 1956; Smith, 1960). More recently it has been suggested that vitamin B_{12} deficiency, in the absence of anaemia, may produce psychiatric symptoms, the diagnosis being made on serum B_{12} levels and therapeutic response following admission to hospital (Smith, 1960; Strachan and Henderson, 1965). Shulman (1967b) suggested that organic confusional states and senile or presenile dementias are the conditions most commonly associated with B_{12} deficiency, though affective disorders may be equally prevalent. Treatment with vitamin B_{12} in such patients (uncontrolled observations) has been said to produce a rapid alleviation of the psychiatric disturbance, and this change is thought to be dramatic in some patients.

Estimates of the prevalence of subnormal serum B_{12} levels in patients admitted to a mental hospital have been made by many workers (Edwin *et al.*, 1965; Hansen *et al.*, 1966; Hutner *et al.*, 1967; Shulman, 1967a; Garden *et al.*, 1968; Murphy *et al.*, 1969). These estimates vary from 0·02% to the high level of 15·4%, but no valid comparison has been made between subjects admitted to hospital and subjects in the community from which they have come. Furthermore, even if it were shown that there is a raised prevalence of low B_{12} levels in hospital admissions, it would still have to be shown that an association with psychiatric symptoms is not due to chance alone (Strachan and Henderson, 1965; Hansen *et al.*, 1966; Murphy *et al.*, 1969).

Community surveys have shown a surprisingly high prevalence of pernicious anaemia (Mosbech, 1953; Girdwood *et al.*, 1967). Though the community survey which preceded the trial described in the present report did not confirm these earlier reports of the prevalence of pernicious anaemia, yet a high prevalence of low serum B_{12} levels without anaemia was found (Elwood *et al.*, 1970) as had been expected (Hansen *et al.*, 1966; Girdwood *et al.*, 1967). It is, however, not known if these low levels have any clinical significance, or how many will go on to develop haematological or psychiatric effects of B_{12} deficiency. From the studies of mental hospital patients quoted above it might be expected that psychiatric ill-health would occur among such subjects and that this would show a beneficial response to B_{12}.

An attempt has therefore been made to determine the psychiatric status of a population sample of elderly people with low serum B_{12} levels, and also the effect thereon of B_{12} supplements by means of a controlled clinical trial. In addition to psychiatric assessment and assessments of general well-being, particular attention was paid to changes in appetite and fatigue, as these symptoms are believed to be influenced by B_{12} therapy.

Method

A 3:20 random sample of subjects aged 65 years and over was chosen from the records of all the practices in a town in Wales (population 42,000). Each subject was invited to co-operate in a haematological survey, the results of which will be reported (Elwood *et al.*, 1970). Eighty-five per cent. of the male and 82% of the female subjects agreed to co-operate, and a sample of venous blood was taken from each. Serum B_{12} was estimated, *Euglena gracilis* Z strain being used (Hutner *et al.*, 1956). Occasional samples were submitted to the laboratory in duplicate, and estimates on these showed that reproducibility was excellent.

The lower limit of the normal range of serum B_{12} levels at the laboratory where these were estimated is 150 pg./ml. Subjects found to have levels below this were asked to co-operate in a clinical trial of the effect of treatment. None of these subjects had anaemia or evidence of B_{12} neuropathy and no subject was known to be taking drugs, such as chlorpromazine, which might have interfered with B_{12} assay, or anticonvulsants, which might reduce the serum B_{12} level. In a parallel study serum pepsinogen estimations were done and only a very small and unimportant difference (.01±.33 μg.) was found between subjects with low B_{12} levels and a representative sample of normal subjects.

Of the subjects with serum B_{12} below 150 pg./ml., half, chosen at random, were given intramuscular hydroxocobalamin (1,000 μg.), twice in the first week and then at weekly intervals for a further four weeks. The other subjects were given intramuscular injections of a matching solution of phenol red (phenylsulphonphthalein 0·075%), the use of which had been approved by the Dunlop Committee.

Each subject was interviewed to assess psychiatric state and general well-being both before and after treatment had been completed. Both interviews were conducted blind with regard to the treatment given. The second interview was also blind with regard to the results of the first interview, and was therefore a fresh assessment. The interview procedure was as follows. Subjects were given a self-administered questionnaire (Goldberg, 1969) which provides information on 60 predominantly psychiatric questions, each answerable by one of four replies which are scored 0, 1, 2, or 3, according to severity. The total score is used as a "global rating" of psychiatric status. The psychiatrist then perused the completed questionnaire and, taking the pattern of replies into account, gave the subject a short and partly structured psychiatric interview. This elicited information on the subject's current and recent psychiatric state, elaborating on any symptoms shown to be present in the questionnaire and evaluating, in particular the subject's state regarding affect, anxiety, sleep, energy/

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fatigue, and appetite. An attempt was made to evaluate the psychiatric state independently of any intellectual deterioration which might have been present in these elderly subjects.

On the basis of this interview subjects were classified into four categories: (0) no detectable psychiatric disorder, (1) subclinical disorder, (2) mild disorder (might require treatment), (3) moderate psychiatric disorder (would probably require treatment), and (4) severe psychiatric disorder (should certainly have treatment). The psychiatric assessment thus obtained was not independent of the questionary but was an attempt to utilize, in addition to questionary replies, other information communicated verbally and non-verbally in a clinical-type interview. The last question on the questionary was "Do you think you are at all ill?" This had five possible graded answers, and was used to obtain an overall self-assessment for each subject.

The interview after treatment was identical except that subjects were also asked whether, since the first interview, there had been any improvement or deterioration in sleeping, energy/fatigue, appetite. The subjects were also asked to illustrate any change, improvement, or deterioration, in general well-being compared with the first interview by choosing a point along a 30-cm. line. The midpoint of this line was explained as representing their state at the first interview, and the distance on one side as representing the degree of any improvement and on the other side deterioration. The midpoint therefore represented no change in general well-being during the trial.

This trial was immediately followed by a long-term morbidity study, and each subject is being seen at frequent intervals to assess further the benefit, if any, of therapy, and to detect the development of a macrocytic anaemia in any subject, should this occur.

Results

The distribution of serum B_{12} levels in the total population sample is shown in Table I. Sixteen men and 28 women had levels below 150 pg./ml. and these were invited to co-operate in the clinical trial. One man and one woman declined and three further women had to be withdrawn during the trial—one had a fall and was admitted to hospital, one had moved out of the area, and one was too deaf to enable valid psychiatric assessments to be made. During the trial there was, on average, a small fall in haemoglobin level, but the difference between the mean changes in those given B_{12} and those given the placebo was very small and not statistically significant (0·01 ± 0·35 g.).

Assessed by questionary, improvement in psychiatric symptoms in those given B_{12} was no greater than in those given the placebo, and the questionary score with the two treatments is similar (Table II). A low mean score occurred in men given the placebo, but this is accounted for by a single subject who produced a very high second questionary score suggesting a pronounced "deterioration." This was thought to have been spurious, as it was not in keeping with the other results for this subject nor with a clinical impression. If his score is omitted the male placebo mean questionary score is similar to that in the other groups.

The subjects' self-assessment as measured by their answers to the last question on the questionary showed no significant difference in the proportion improved with the two treatments (Table II). Similarly, the linear self-assessment (Table III) showed no difference between the groups in the proportion improved. A comparison of the two groups with regard to sleep, energy/fatigue, and appetite is shown in Table IV. A high proportion reported benefit with treatment, but there were no significant differences between the groups.

Fourteen subjects were thought to have some psychiatric abnormality as assessed by the first interview—10 in category 1, 2 in category 2 and 2 in category 3. At the second interview only two subjects were judged to be in category 1 and one in category 2. The changes in each group are set out in Table V. No subject deteriorated, but while a third of the subjects improved, again there was no significant difference in the two treatment groups.

TABLE I.—*Distribution of Serum B_{12} Levels in the Total Population Sample of Subjects 65 Years of Age and Over*

Serum B_{12} (pg./ml.)	No. of Subjects	
	Males	Females
Under 100	1	7
100–149	15	21
150–199	12	18
200–249	31	29
250–299	21	20
300–349	4	11
350–399	4	3
400–449	2	12
450–499	—	3
500 and over	3	8
Total	93	132

TABLE III.—*Linear Self-assessment Scale. Number Improved and Unchanged, and Mean Improvement for All Subjects Expressed in cm. (see Text)*

Sex	Given B_{12}			Given Placebo		
	Improved	No Change	Overall Mean Improvement	Improved	No Change	Overall Mean Improvement
Male	6	2	4·58	5	2	4·25
Female	9	3	3·87	9	3	4·43
Total	15	5	4·3	14	5	4·35

TABLE IV.—*Changes in Appetite, Energy/Fatigue, and Sleep*

Symptom	Given B_{12}						Given Placebo					
	Males		Females		Total		Males		Females		Total	
	+	O	+	O	+	O	+	O	+	O	+	O
Appetite	3	5	2	10	5	15	2	5	0	12	2	17
Energy/fatigue	2	6	8	4	10	10	4	3	6	6	10	9
Sleep	2	6	3	9	5	15	2	5	2	10	4	15

+ = Indicates an improvement. O = Indicates no change.

TABLE II.—*Number of Subjects who Showed an Improvement, No Change, or a Deterioration in Scores with Treatment, Assessed by Questionary. Number who Changed on the Self-assessment Question Shown in Parentheses*

Sex	Given B_{12}				Given Placebo			
	Improved	No Change	Deteriorated	Overall Mean Improvement in Score	Improved	No Change	Deteriorated	Overall Mean Improvement in Score
Male	5 (2)	0 (4)	3 (2)	7·4	6 (2)	0 (3)	1 (2)	0·4 7·5*
Female	8 (5)	2 (6)	2 (1)	6·8	10 (3)	2 (7)	0 (2)	6·9
Total	13 (7)	2 (10)	5 (3)	7·1	16 (5)	2 (10)	1 (4)	4·5 7·2*

None of the differences is significant.
*Mean excluding one subject with spuriously high second score.

TABLE V.—*Changes in Global Rating Based on Psychiatric Interview (see Text)*

Sex	Given B_{12}		Given Placebo	
	Improved	No Change	Improved	No Change
Male	2	6	0	7
Female	5	7	6	6
Total	7	13	6	13

Overall female improvement exceeds male improvement significantly. P (exact test) = 0·039, otherwise no differences are significant.

Most of the subjects showed some improvement at the second evaluation. This is not unexpected, nor was it unexpected that in general no appreciable deterioration was detected during the trial. On the questionary assessment 29 subjects improved while only six deteriorated. On the self-assessment question 12 improved and none deteriorated. On the linear self-assessment scale 29 improved and none deteriorated, and in the clinical assessment 13 improved and none deteriorated. None of these tests, however, produced evidence that subjects given B_{12} improved more than those given the placebo.

Discussion

Vitamin B_{12} appears to be given not infrequently in conditions other than those associated with B_{12} deficiency (International Medical Statistics, 1969; Ellis *et al.*, 1970), its use being based on the belief that it has a beneficial effect on such symptoms as appetite, fatigue, and sleep and possibly on certain aspects of mental health. This last may be based in part on the clinical observations quoted above. This study does not show that there is any special beneficial effect from the administration of B_{12} to subjects with low B_{12} levels. Previous observations on 828 consecutive admissions to the mental hospital serving the area in which the present trial was conducted disclosed several patients with pernicious anaemia, but these patients provided no evidence of an association between serum B_{12} and severity of psychiatric illness, E.E.G. changes, or psychometry; nor was there any association between B_{12} level on admission and the proportion of the 828 patients still in hospital six months later (S. Nam, private communication, 1969).

These observations and the results of the present trial do not, of course, exclude the existence of a psychiatric syndrome resulting from B_{12} deficiency, though they do perhaps emphasize its rarity. Murphy *et al.* (1969) doubt that it is even common enough to justify, at present, routine serum B_{12} assays on mental hospital admissions. During the trial a high proportion of the subjects showed evidence of an improvement with treatment whether B_{12} or the placebo was given, while in general no appreciable deterioration was detected. As the second interviews were all conducted blind, and as there was a long interval between the first and second tests, probably most subjects did actually improve in psychiatric state and general well-being. In the absence of evidence of an effect of B_{12} this improvement must simply represent the therapeutic effect of the interest and concern shown by a team of doctors, nurses, and research workers, who paid repeated visits to the homes and gave a course of injections over several weeks. It is of interest, however, that whereas a third of all the subjects improved there was a significant difference between the sexes; only 2 of the 15 men improved, while 11 of the 24 women did so. This difference may well be related to the fact that 17 of the 24 women were widowed and eight lived alone, while only 2 of the 15 men were widowers and none lived alone.

Another methodological aspect of the trial is the relation between the tests. The correlation between independent measures of the same abnormality can be taken as a measure of their validity. Subjects grouped for the degree of improvement assessed by the clinical interviews, the mean improvement in the questionary score, and the mean change in the linear self-assessment measurement are shown in Table IV. There is a concordance between the clinical rating and the questionary score, and assuming that the distribution of changes in questionary score is gaussian, this concordance is statistically significant. This, however, might be regarded as unremarkable, as it can be argued that the tests as conducted were not independent of each other. Nevertheless the linear self-assessment and the psychiatric rating, which are independent of each other, also show significant concordance.

TABLE VI.—*Concordance Between Tests. Mean Changes in Questionary Score and in Linear Self-assessment in Subjects Grouped by Change in Psychiatric Rating Based on Clinical Interviews*

Psychiatric Rating	Mean Improvement in: Questionary Score	Mean Improvement in: Linear Self-assessment
Nil	2·9 *	3·0 cm. *
One category	11·6 *	6·5 cm. *
Two categories	27·0 *	7·7 cm. *

*Differ significantly (*t* test) at P<0·05.

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Familial Vitamin B_{12} Malabsorption

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One of us (7) has formerly described 10 patients suffering from chronic relapsing megaloblastic anaemia with onset in infancy and childhood. Eight of the patients also had permanent proteinuria without other signs of impaired renal function. There was a familial occurrence of the syndrome: chronic relapsing megaloblastic anaemia and permanent proteinuria. These patients seemed to represent a specific, selective vitamin B_{12} malabsorption disease which had not been recognized as such before. The disease (and the syndrome) was assumed to be congenital and hereditary.

The purpose of the present work has been to re-examine the 10 patients in order to attempt clarifying the pathogenesis and for this object we have used radioactive Co-labelled vitamin B_{12}.

Methods

The absorption of radioactive B_{12} has been measured by the method of Schilling (9). The assays have been performed in the standard manner using Co^{58} labelled vitamin B_{12} made by Philips "Duphar". Each oral test-dose has contained 1 microgram vitamin B_{12} with a specific activity of 1 $\mu c./\mu g$. The radioactivity has been counted in urine collected in 24 hours.

The presence of intrinsic factor: First an ordinary Schilling test has been performed. Then the test has been repeated after an intrinsic factor concentrate was added to the oral test-dose. Finally the intrinsic factor activity of the gastric juice has been tested in bioassays on proved cases of pernicious anaemia (PA). From each patient a sample of 80 ml. neutralized gastric juice was administered by stomach tube to a PA patient immediately after an oral test-dose Co^{58} B_{12} and the amount of B_{12} radioactivity appearing in the urine during the next 24 hours was determined.

The intrinsic factor concentrate (IFC) was prepared by Abbot Laboratories from desiccated hog stomach. The IFC had been proved effective in assays on known PA.

The gastric juice from our patients was collected after intramuscular injection of 0.5 mg. histamine and filtered through gauze. pH was immediately adjusted to 10 with N NaOH to inactivate pepsin and after 20 minutes titrated to pH 7 with N HCl. The juice was kept frozen at —20 °C until just before use.

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Antibiotics, aureomycin or achromycin 250 mg., were given perorally 3 times daily for 5 days. The SCHILLING tests were performed after 4 days of treatment.

Prednisone, 10 mg. was given perorally 3 times daily for 6 days.

The SCHILLING tests were performed after 5 days of treatment.

Other laboratory methods were those described by IMERSLUND (7).

Material

The material consists of 6 males and 4 females. In the present paper they have the same designation as before with Roman numbers. They belong to 6 different sibships and families designated as A, B, C, D, E and F (table I). Only siblings are affected, no parents or other relatives. No consanguinity has been demonstrated between the parents. However, 3 couples have their origin from relatively isolated valleys in Norway.

The onset of symptoms was from 5–6 months to 4 years of age (table I).

Table I

Age when symptoms started and when anaemia and proteinuria were discovered.

Family	Patient No.		Age when symptoms started years	Age when anaemia was discovered years	Age when proteinuria was discovered years
A	I	♂	$1^{3}/_{12}$	$1^{5}/_{12}$	$2^{3}/_{12}$
	II	♂	$^{5}/_{12}$	$^{7}/_{12}$	$^{5}/_{12}$
B	III	♂*	4	$4^{1}/_{12}$	
C	IV	♂	$1^{9}/_{12}$	2	2
	V	♀	$1^{2}/_{12}$	$1^{3}/_{12}$	$1^{9}/_{12}$
	VI	♀	$1^{3}/_{12}$	$1^{6}/_{12}$	$1^{4}/_{12}$
D	VII	♀	$1^{3}/_{12}$	$1^{9}/_{12}$	$1^{9}/_{12}$
	VIII	♂	1	$1^{2}/_{12}$	1
E	IX	♀	$1^{10}/_{12}$ ($^{12}/_{12}$?)	2	2
F	X	♂	$1^{3}/_{12}$	$1^{6}/_{12}$	

* A sister died from megaloblastic anaemia at the age of $3^{9}/_{12}$ years.

Symptoms and signs were such as are seen in vitamin B_{12} deficiency: Pallor, weakness, irritability, gastrointestinal symptoms, glossitis or atrophic tongue, loss of appetite, tendency to constipation or slight diarrhea. Slight or doubtful neurological changes were observed in three patients and premature greying of the hair in one.

Haematological findings: The anaemia was macrocytic with Price-Jones curves of asymmetric heterogeneous type similar to those seen in pernicious anaemia. Tendency to moderate leucopenia, granulocytopenia and occasional thrombopenia was observed. The serum iron was with a few exceptions increased, the icterus index normal or slightly increased. The bone marrow showed typical megaloblastosis with giant myelocytes and "stabcells".

Findings in the urine: Eight of the 10 patients (table I) had permanent proteinuria (from trace to 2%) with minimal microscopic findings. By the concentration tests specific gravity was 1027—1036. Urea and creatinine clearances were within normal range.

Urinary tract anomalies: Five of the 10 patients had urinary tract anomalies of a relatively homogenous appearance which was regarded as representing different stages of duplication of the urinary pelvis and ureter, from slight to complete.

Gastric juice: Free hydrochloric acid was present in all aspirated Ewald's test meals except once in one patient and always in gastric juice aspirated after injection of

histamine. The quantities of microbiologically determined vitamin B_{12} binding factor in the gastric juice were normal.

Perorally administered small amounts of vitamin B_{12} + normal pooled human gastric juice did not produce any rise of haemoglobin and red blood cells (experiments carried out in 3 patients).

Vitamin B_{12} in plasma examined partly in remission and partly in relapse was decreased and varied between 15—448 $\mu\mu g./ml$.

Stools: Several examinations for ova and parasites were negative. Fat balance tests showed normal absorption of fat, varying between 91—99%.

Glucose tolerance curves were normal.

Gastrointestinal X-rays after Ba constrast were normal.

Response to treatment with vitamin B_{12}: Intramuscular injections of vitamin B_{12} induced typical haematological reaction and remission. Monthly injections of 50—100 micrograms of vitamin B_{12} have maintained remission and well-being until the present time. Without specific antipernicious treatment the anaemia has always relapsed.

Present Investigation

The age of the patients varied from 7—25 years (Table II). They were well. Growth and development were normal. Thus 7 of the [illegible] taller than the average and 3 of them shorter, the shortest being in the 25 p[illegible]. The intelligence was normal judged by their achievements in school and later on. No definite abnormalities were detected at the physical examination except for premature greying of the hair in the oldest patient. There was no hypertension or enlargement of the heart. There were no neurological symptoms or signs.

The haemoglobin, red and white blood cell count and the differential count were within normal range.

Findings in the urine: Eight of the 10 patients had proteinuria as before (from trace to 1‰) with minimal microscopic findings and no growth by bacteriological examination. At the electrophoretic examinations the urinary proteins consisted mainly of albumin.

Renal function: By concentration tests the specific gravity rose to 1029—1038. The serum creatinine was normal.

Gastric juice: The volume was normal when compared with a material of healthy individuals from the same age groups. pH varied between 1—2.

Serum proteins were normal (electrophoresis).

Results

The results of the SCHILLING tests are seen in table II. The data represent the percentages of the oral test-dose Co^{58} B_{12} excreted in the urine in 24 hours. Without intrinsic factor material the patients excreted between 0 and 1.4 % of the test-dose given. When IFC was added to the test-dose there was no significant increase in the excretion. There was no increase in the excretion after sterilization of the gut with achromycin (given to 5 patients). There was no increase in the excretion after prednisone therapy (given to 3 patients).

Table II

The results of the SCHILLING tests in our patients.

Pat. No.	Age years	% excretion of oral test dose $Co^{58}B_{12}$ in urine in 24 hours			
		Without IFC	With hog IFC	After achromycin therapy	After prednisone therapy
I	25	1.2	1.0		
II	7	1.3	1.0	0.4	
III	19	0.6	1.0		
IV	19	0	0		
V	14	0	0	0.2	
VI	14	0.2	0.2		0
VII	19	0.1	0.3	0.4	0.1
VIII	14	0.2	0.2		
IX	12	1.4	0.4	0.5	
X	11	0	0.3	0.3	0.3

Table III

The results of the SCHILLING tests in pernicious anaemia patients.

PA	% excretion of oral test dose $Co^{58}B_{12}$ in urine in 24 hours		
	Without IFC	With hog IFC	With gastric juice from our patients
T. H.	0.8	10.0	10.4 (No. I)
L. H.	0.2	14.7	12.0 (No. II)
S. M.	1.3	13.6	19.0 (No. III)
F. N.	0.8	13.8	17.4 (No. IV)
T. H.	0.8	10.0	12.0 (No. V)
F. N.	0.8	13.8	15.0 (No. VI)
T. H.	0.8	10.0	14.0 (No. VII)
R. S.	1.5	16.6	14.0 (No. VIII)
J. M.	0.9	16.5	11.0 (No. IX)
R. T.	0.8	13.0	11.8 (No. X)

Table III demonstrates the intrinsic factor activity of the patients "gastric" juice when tested on different PA patients. Without administration of intrinsic factor the PA patients excreted 0.2—1.5 % of the test-dose given. When hog IFC was added the excretion rose to 10—16.6 %. When the PA patients were given the test-dose $Co^{58}B_{12}$ + the gastric juice from our patients the PA patients excreted between 10.4—19.0 % of the test-dose. Thus the intrinsic factor activity in the gastric juice was normal when tested on proved pernicious anaemia.

At all investigations the results were the same in the patients with proteinuria as in those without. Treatment with antibiotics had no effect on the occurrence of proteinuria.

Discussion

The vitamin B_{12} deficiency is demonstrated by the megaloblastic bone marrow, the low level of plasma vitamin B_{12} and the therapeutic response to intramuscular injections of vitamin B_{12}. The results of the SCHILLING test demonstrate that the cause of the vitamin B_{12} deficiency is decreased absorption from the gastrointestinal tract.

With regard to the pathogenesis we have to consider the following known causes of impaired absorption of vitamin B_{12}:

1. *Lack of intrinsic factor* can be excluded as the gastric intrinsic factor activity is normal in bioassays on proved PA, a finding which fits with the negative response in our patients after IFC was added to the oral test-dose Co^{58} B_{12}.

2. *Utilization of the vitamin by intestinal micro-organisms and parasites* can be excluded. There was no increase in the absorption of the radioactive labelled vitamin B_{12} after treatment with antibiotics. No ova or parasites have been found in the stools.

3. *Gross pathological changes in the gastrointestinal tract* including strictures, anastomoses, blind loops and diverticulosis can be excluded because of the normal radiological findings and because the resorption was not improved after treatment with antibiotics.

4. *Coeliac disease or idiopatic steatorrhea* can be excluded because of normal resorption of glucose and fat. In addition we have the clinical evidence against the presence of multiple absorption deficiencies, the normal growth and development when treated only with parenteral injections of vitamin B_{12}.

5. *Immunological mechanism* are still an obscure cause of vitamin B_{12} deficiency. However, the fact that the vitamin B_{12} absorption was not improved after prednisone therapy points against immunological mechanisms playing any role.

As known causes of impaired absorption of vitamin B_{12} seem to be excluded, it seems reasonable to assume that the patients are lacking some specific intestinal factor (or factors) necessary for the normal absorption of vitamin B_{12}. Thus the so-called releasing factor demonstrated in the intestinal wall of the rat (3, 6) may also be necessary in man. The lack of such a factor which probably is of enzymatic nature could be the cause of the vitamin B_{12} malabsorption in our patients.

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Clark and Booth (1) and Dallman and Diamond (4) demonstrated lack of vitamin B_{12} absorption and vitamin B_{12} deficiency in 2 children aged 13 months and 3 years respectively after extensive resection of the distal small intestine in the newborn period. Apparently the clinical symptoms suggesting vitamin B_{12} deficiency in these patients had started at about 10 and 18 months of age. These observations support the previous assumption about the malabsorption being congenital in our patients, as the symptoms of vitamin B_{12} deficiency in most of them started in the same age group as in the afore-mentioned patients. The onset of symptoms probably depends upon the time required to empty the congenital stores of vitamin B_{12}. The situation seems analogous to the well known delayed development of vitamin B_{12} deficiency in patients after total gastrectomy. The fact that the results of the present investigation have been the same in the patients with proteinuria as in those without may fit in with the previous hypothesis that the proteinuria is an associated genetic phenomenon without primary pathogenetic importance for the vitamin B_{12} malabsorption.

It seems evident that our patients represent a new specific group of vitamin B_{12} malabsorption disease. To this group may belong the cases described by Gråsbeck et al. (5) as well as those described by Colle et al. (2) and by Lamy et al. (8). To the same group may also belong many of the incompletely examined cases formerly described as pernicious anaemia in infants and children.

Summary

A report is given on the results of the Schilling test in 10 previously described patients suffering from an apparently selective and specific vitamin B_{12} malabsorption disease. Without intrinsic factor material the urinary excretion of Co^{58} B_{12} was between 0 and 1.4% of the test-dose administered. There was no significant increase in the excretion when intrinsic factor material was added to the test-dose Co^{58} B_{12} neither after treatment with antibiotics (achromycin given to 5 patients) nor after prednisone (given to 3 patients). The intrinsic factor activity of the patients' gastric juice was normal when tested on patients with pernicious anaemia.

Résumé

Rapport sur les résultats obtenus à l'aide du test de Schilling chez 10 malades décrits auparavant déjà, souffrants d'une altération apparamment sélective et spécifique de l'absorption de la vitamine B_{12}. L'excrétion urinaire de la vitamine B_{12}-Co^{58} était de 0-1,4% de la dose-épreuve appliquée sans le facteur intrinsèque. Aucune augmentation significative de l'excrétion ne pouvait être observée après avoir ajouté le facteur intrin-

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sèque à la dose-épreuve de vitamine B_{12}-Co^{58}, ni après traitement par les antibiotiques (achromycine chez 5 malades), ni après application de prednisone (chez 3 malades). L'activité du facteur intrinsèque du suc gastrique des malades était normale (dosage chez des malades atteints d'une anémie pernicieuse).

Zusammenfassung

Es wird über die Ergebnisse mit dem SCHILLING-Test bei 10 früher beschriebenen Fällen von scheinbar selektiver und spezifischer Vitamin B_{12}-Resorptionsstörung berichtet. Ohne Intrinsic-Faktor betrug die Urinausscheidung von Co^{58}-B_{12} 0–1.4% der zugeführten Testdosis. Es fand sich kein signifikanter Anstieg der Ausscheidung, wenn Intrinsic-Faktor der Testdosis von Co^{58}–B_{12} zugefügt wurde, weder nach Behandlung mit Antibioticis (Achromycin bei 5 Patienten), noch nach Verabreichung von Prednison (bei 3 Patienten). Der Magensaft der Patienten zeigte normale Intrinsic-Faktor-Aktivität bei Prüfung an Kranken mit perniziöser Anämie.

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CONCERNING THE QUESTION OF SIDE EFFECTS OF VITAMINS IN THE TREATMENT OF PATIENTS WITH ATHEROSCLEROSIS OF THE CORONARY ARTERIES

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In the complex treatment of patients with coronary atherosclerosis, definite significance is attached to the use of vitamins (especially vitamins B_6 and B_{12}, ascorbic acid, and others.)

On the other hand, as the results of our research have shown, in a few patients with atherosclerosis of the coronary arteries in stages and III (according to the classification of A. L. Myasnikov [17]), prolonged use of vitamins does not produce an effect or provokes side effects.

This fact seems important to us: that side effects of vitamin therapy were observed by us at the beginning of treatment or after repeated courses of therapy using vitamins not only at a maximum, but also at optimum dosage. Therefore, during the use of vitamin B_{12} in a dose of 200 μg every twenty-four hours, more frequent and intensified bouts of stenocardia were noted in a few patients with atherosclerosis of the coronary vessels. Other authors [3] have observed an analagous side effect of vitamin B_{12}. T. D. Telegina [23] observed 3 patients with cardio-vascular disease, in whom headache and a crushing pain behind the hest appeared after each injection; pain in the stomach appeared in one.

In a few patients pronounced allergic reactions were noted after repeated se of vitamin B_{12}, clear up to development of anaphylactic shock 1,2 as a result of sensitivity to the preparation. An intradermal test for vitamin B_{12} made on them proved positive (hyperemia, swelling or blisters.) The prolonged use of vitamin B_{12} in large doses favors the beginning and development of the blastomatosic process, and therefore the evidence recommends that treatment by this vitamin be restricted in oncologic practise.

Side effects of vitamin B_1 during the course of its use were observed most often of all and appeared in the form of allergic reaction -- dermatitis, exanthem with an itching factor, a rise in body temperature, relapsed Quinche's edema [5, 12, 16] , and also in the form of anaphylactic shock [10, 19] and myocarditis. A. S. Lopatin [10] described the development, in a patient after 1 intra-muscular injections of vitamin B_6 in a 1.0 ml .5% solution, of phenomena clinically similar to the picture of acute stomach. He observed in one patient the development of an anaphylactic reaction (hyperemia of the integument, difficulty in breathing, paroxysmic tachycardia) after a single dose of vitamin B_{15}. The use of repeated courses of therapy with vitamin B_{15} in a few patients with ischemic disease of the heart produced more frequent bouts of stenocardia [6].

In a few patients with coronary atherosclerosis, we noted pronounced side effects of nicotinic acid and its derivitives (nicatol) during internal use in gradually increasing doses (first 2 days at 0.25 g 3 times a day, then 0.5 g 3 times a day) in the form of congestion, sharp fever, a fine petechial rash, a harsh skin itch, tachycardia, intensified extrasystoles, more frequent and intensified bouts of stenocardia, with he development in one patient of myocardial infarction [9]. After

intravenous introduction of a solution of nicotinic acid, the appearnce of a bout of paroxysmic tachycardia [13] was noted. According to the data of the literature [20-22] use of nicotinic acid is contraindicated by tyrotoxicosis, neurosis, nephrosclerosis, pronounced tachycardia, and polytropic extrasystole. A few authors consider [11] that a surplus of nicotinic acid is harmful, because the organism eliminates it in methylated form, which leads to the impoverishment of the organism in methionine and choline.

At the present time the opinion has become ingrained that the use of large doses of ascorbic acid is safe. Meanwhile, ever increasing information is being accumulated which testifies to the fact that ascorbic acid can produce side effects even at optimum dosage during prolonged use. Therefore, according to the data we obtained, repeated courses of therapy with ascorbic acid at a dose of 0.5 g 3 times a day for a month according to the method proposed by A. L. Myasnikov [18])provoked in three patients with atherosclerosis of the coronary arteries in stages I and III the appearance of glucosuria and moderate hyperglycemia, i.e., a light form of sugar diabetes. Analagous side effects of ascorbic acid during its use in large doses for a prolonged length of time have been noted by other authors [14, 24], who consider that ascorbic acid in large doses changes the permeability of the vessels of the kidneys, can lead to a disturbance in the function of the islets of Langerhans, as a result of which sugar diabetes develops. Glatzel [29] observed, as did we, the development of glucosuria during use of ascorbic acid at 1.0-1.5 g every 24 hours. M. F. Merezhinski [15] noted that ascorbic acid in large doses shows a stimulating influence on the production of ortisone and other steroid hormones which, under certain conditions,

can lead to damage of the glomeruli of the kidneys and development of arterial hypertonia.

In connection with the stated treatment by ascorbic acid in large doses, the maintenance of sugar in the urine and blood should be conducted under systematic control, and also the level of arterial pressure should be kept under control. It is possible to make a practical conclusion about this: that to prescribe ascorbic acid in large doses is contraindicated for patients with sugar diabetes and hypertonic disease. Widenbauer [30] noted a toxic effect of ascorbic acid during continuous use of it in doses of 550-1000 mg in the course of 5-6 months. According to the data of R. V. Chagovits [25], during the introduction of large doses of ascorbic acid in an experiment a significant change in tissue dehydrase occurred. E. F. Shampai [27], E. F. Shampai and Yu. V. Khmelevski [28] report that the use of ascorbic acid in a dose of .0 g and higher every 24 hours exerts a toxic effect on the organism of man, producing first of all a disturbance in the function of the sexual glands.

Therefore, it is possible to conclude that during prescription of vitamin therapy to patients with coronary atherosclerosis, a reduction in the dose of the preparation should be aimed for, especially during treatment of elderly and senile patients, and also a reduction in the courses of therapy and a lengthening in the intervals between them without detriment to the medicinal effect should be aimed for. The removal of factors that cause deterioration in the absorption and assimilation of vitamins, reducing their activity and stability, has an important significance (timely and active treatment of diseases of the gastrointestinal tract and liver, the clearing of chronic centers of infection,

strict grounds for prescribing antibiotics, sulfanilamids, and other reparations that depress intestinal flora.) A deterioration in the maintenance or an absence of free hydrochloric acid in the contents of the stomach also worsens the assimilation of vitamins. Therefore, for patients with hypo- or anacidic gastritis, it is necessary to prescribe gastric juices or solutions of hydrochloric acid with pepsin simultaneously with the vitamins. On the other hand, it should be remembered that prolonged use of vitamin B_6 can promote an increase in the acidity of the stomach contents. In the presence of enteritis and chronic centers of infection, vitamins are assimilated better during parenteral use; therefore, during aggravation of the stated diseases, it is advisable to apply them in the form of injections.

During vitamin therapy with stabilized vitamins, a sufficient quantity of albumen should be prescribed [4, 26], and during treatment with nicotinic acid -- preparations containing active methylene groups in sufficient quantity (vitamin B_{15}, methionine, choline-chloride).

We believe that with the goal of intensifying and prolonging the medicinal effect of vitamins and with the warning about their side effects during treatment of patients with coronary atherosclerosis, it is advisable to use, in optimal dosages, a wide range of vitamins possessing a synergistic and mutually potentiating effect. We confirmed the more pronounced medicinal effect of the vitamin complex (ascorbic, folic acid, riboflavin, rutin, vitamin B_6) during regulation of its use in patients with coronary atherosclerosis in stages I and III in the form of repeated monthly courses (an average of 3-4 courses per year).

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К ВОПРОСУ О ПОБОЧНОМ ДЕЙСТВИИ ВИТАМИНОВ ПРИ ЛЕЧЕНИИ БОЛЬНЫХ АТЕРОСКЛЕРОЗОМ КОРОНАРНЫХ АРТЕРИЙ

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В комплексном лечении больных коронарным атеросклерозом определенное значение придается применению витаминов (особенно витаминов B_6 и B_{12}, аскорбиновой кислоты и др.).

Однако, как показали результаты наших исследований, у некоторых больных атеросклерозом коронарных артерий I и III стадий (по классификации А. Л. Мясникова [17]) длительное применение витаминов не давало эффекта или вызвало побочные действия.

Нам представляется важным тот факт, что побочные действия витаминотерапии наблюдались нами в начале лечения или после повторных курсов терапии при применении витаминов не только в максимальной, но и в оптимальной дозе. Так, при применении витамина B_{12} в дозе 200 *мкг* в сутки [8] отмечалось учащение и усиление приступов стенокардии у некоторых больных атеросклерозом коронарных сосудов. Аналогичное побочное действие витамина B_{12} наблюдали и другие авторы [3]. Т. Д. Телегина [23] наблюдала за 3 больными сердечно-сосудистыми заболеваниями, у которых после каждой инъекции появлялась головная боль, давящая боль за грудиной; у одной больной возникла тупая боль в животе. У некоторых больных после повторного применения витамина B_{12} отмечались выраженные аллергические реакции вплоть до развития анафилактического шока [1, 2] вследствие сенсибилизации к препарату. Произведенная у них внутрикожная проба с витамином B_{12} оказалась положительной (гиперемия, припухлость или пузыри). Длительное применение витамина B_{12} в больших дозах способствует возникновению и развитию бластоматозного процесса, в связи с чем рекомендуется ограничивать показания к лечению этим витамином в онкологической практике [7].

Побочное действие витамина B_1 чаще всего наблюдалось при курсовом его применении и проявлялось в виде аллергических реакций — дерматитов, экзантем с зудящим компонентом, повышения температуры тела, рецидивирующего отека Квинке [5, 12, 16], а также в виде анафилактического шока [10, 19] и миокардита. А. С. Лопатин [10] описал развитие у больного после 2 внутримышечных инъекций витамина B_6 по 1,0 *мл* 2,5% раствора явлений, клинически сходных с картиной острого живота. Он наблюдал у одного больного

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развитие анафилактической реакции (гиперемия кожных покровов, затруднение дыхания, пароксизмальная тахикардия) после однократного приема витамина B_{15}. Применение повторных курсов терапии витамином B_{15} у некоторых больных ишемической болезнью сердца вызывало учащение приступов стенокардии [6].

Мы отмечали у некоторых больных коронарным атеросклерозом выраженное побочное действие никотиновой кислоты и ее производных (никатола) при применении внутрь в постепенно возрастающих дозах (первые 2 дня по 0,25 *г* 3 раза в день, затем по 0,5 *г* 3 раза в день) в виде приливов, резкого ощущения жара, мелкопетехиальной сыпи, резкого кожного зуда, тахикардии, усиления экстрасистолии, учащения и усиления приступов стенокардии с развитием у одного больного инфаркта миокарда [9]. После внутривенного введения раствора никотиновой кислоты отмечено появление приступа пароксизмальной тахикардии [13]. Согласно данным литературы [20—22], противопоказанием к применению никотиновой кислоты являются тиреотоксикоз, невроз, нефросклероз, выраженная тахикардия и политопная экстрасистолия. Некоторые авторы считают [11], что избыток никотиновой кислоты вреден, так как организм выводит ее в метилированном виде, что приводит к обеднению организма метионином и холином.

В настоящее время укоренилось мнение о безопасности применения больших доз аскорбиновой кислоты. Между тем накапливается все больше фактов, свидетельствующих о том, что аскорбиновая кислота даже в оптимальной дозировке при длительном применении может оказать побочное действие. Так, согласно полученным нами данным, повторные курсы терапии аскорбиновой кислотой в дозе 0,5 *г* 3 раза в день в течение месяца (по методике, предложенной А. Л. Мясниковым [18]) у 3 больных атеросклерозом коронарных артерий I и III стадий вызвали появление глюкозурии и умеренной гипергликемии, т. е. легкой формы сахарного диабета. Аналогичные побочные влияния аскорбиновой кислоты при применении ее в больших дозах на протяжении длительного времени отмечены и другими авторами [14, 24], которые считают, что аскорбиновая кислота в больших дозах изменяет проницаемость сосудов почек, может привести к нарушению функции островков Лангерганса, вследствие чего развивается сахарный диабет. Glatzel [29] наблюдал, как и мы, развитие глюкозурии при применении аскорбиновой кислоты по 1,0—1,5 *г* в сутки. М. Ф. Мережинский [15] отмечает, что аскорбиновая кислота в больших дозах оказывает стимулирующее влияние на образование кортизона и других стероидных гормонов, что при известных условиях может привести к повреждению гломерул почек и развитию артериальной гипертонии.

В связи с изложенным лечение аскорбиновой кислоты в больших дозах следует проводить под систематическим контролем содержания сахара в моче и крови, а также под контролем уровня артериального давления. Можно сделать практический вывод о том, что больным сахарным диабетом и гипертонической болезнью назначать аскорбиновую кислоту в больших дозах противопоказано. Widenbauer [30] отметил токсическое действие аскорбиновой кислоты при непрерывном применении ее в дозе 550—1000 *мг* в течение 5—6 месяцев. Согласно данным Р. В. Чаговца [25], при введении больших доз аскорбиновой кислоты в эксперименте происходит значительное изменение тканевых дегидраз. Е. Ф. Шамрай [27], Е. Ф. Шамрай и Ю. В. Хмелевский [28] сообщают, что применение аскорбиновой кислоты в дозе 1,0 *г* и выше в сутки оказывает токсическое действие на организм человека, вызывая прежде всего нарушение функции половых желез.

Таким образом, можно заключить, что при назначении витаминотерапии больным коронарным атеросклерозом следует стремиться к снижению дозы препаратов, особенно при лечении больных пожилого и старческого возраста, а также к сокращению курсов терапии и удлинению промежутков между ними без ущерба для лечебного эффекта. Важное значение имеет устранение факторов, ухудшающих всасывание и усвоение витаминов, снижающих их активность и устойчивость (своевременное и активное лечение заболеваний желудочно-кишечного тракта и печени, санация хронических очагов инфекции, строгое обоснование при назначении антибиотиков, сульфаниламидов и других препаратов, угнетающих кишечную флору). Ухудшает усвоение витаминов также снижение содержания или отсутствие в желудочном содержимом свободной соляной кислоты. Поэтому больным с наличием гипо- или ахилидного гастрита необходимо одновременно с витаминами назначать желудочный сок или раствор соляной кислоты с пепсином. Однако следует помнить, что длительное применение витамина B_6 может способствовать повышению кислотности желудочного содержимого. При наличии энтерита и хронических очагов инфекции витамины лучше усваиваются при парентеральном применении, поэтому при обострении указанных заболеваний их целесообразно применять в виде инъекций.

При витаминотерапии в качестве стабилизаторов витаминов следует назначать достаточное количество белков [4, 26], а при лечении никотиновой кислотой — препараты, содержащие в достаточном количестве активные метильные группы (витамин B_{15}, метионин, холин-хлорид).

Мы полагаем, что с целью усиления и пролонгирования лечебного эффекта витаминов и предупреждения их побочного действия при лечении больных коронарным атеросклерозом целесообразно применять в оптимальной дозировке широкий комплекс витаминов, обладающих синергизмом и взаимопотенцированием действия. Нами подтвержден более выраженный лечебный эффект комплекса витаминов (аскорбиновая, фолиевая кислоты, рибофлавин, рутин, витамин B_6) при регулярном его применении у больных коронарным атеросклерозом I и III стадий в виде повторных месячных курсов (в среднем 3—4 курса ежегодно).

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CONTRIBUTION OF VITAMIN B_{12} TO THE FORMATION OF Fe^{55}-PORPHYRIN FROM INORGANIC Fe^{55} [1]

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It is a well known fact that the decrease in red blood cell counts and hemoglobin content occurs in vitamin B_{12}-deficiency of animals. In consideration of this fact, the authors (1) have studied the relationship between B_{12}-deficiency in cattle and B_{12}-producing intestinal microflora, as well as that between B_{12}-nutrition and carboxylase formation in animals (2). The authors (3) have observed that the bound B_{12} in bovine blood occurs mostly in erythrocytes, and suggested that the vitamin may also directly contribute to the biosynthesis of iron-porphyrin from inorganic iron.

We (4) have further carried out several experiments of injecting inorganic Fe^{55} in B_{12}-deficient rats and tested the *in vitro* synthesis (5) of Fe^{55}-porphyrin by bone marrow homogenate from the animal which had received inorganic Fe^{55}. The results of this experiment will be reported in this paper.

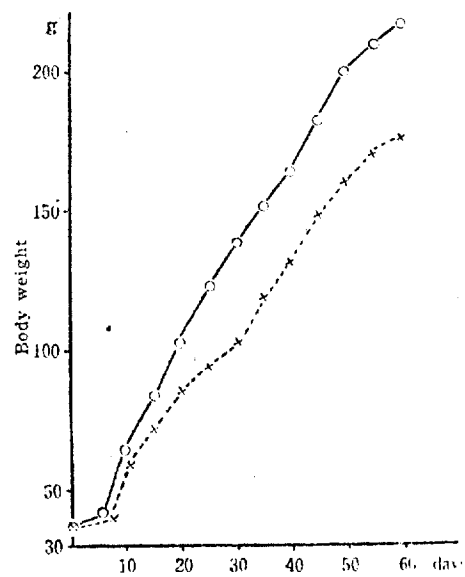

FIG. 1 *Growth Curve of the B_{12} Deficient Rats*

o The group received 0.2 γ B_{12} per head daily.

× B_{12}-deficient Group.

EXPERIMENTAL

1. Production of B_{12}-deficiency in Rats.

19 Young rats weighing 35—45 g, born from female rats kept on B_{12}-deficient diets, were used in the experiments. The animals were divided at random into two groups, one of which was fed completely B_{12}-deficient diets. The other group served as control animals receiving orally 0.2 γ

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of the vitamin per head daily.

The B_{12}-deficient diet was composed of, in per cent, potato starch, 57.7; wheat flour, 7.0; purified casein, 13.0; soy bean meal, 10.0; soy bean oil plus cod liver oil (10 : 1), 7.0; McCollum salt mixture, 4.0; choline, 0.1; cystine, 0.2; vitamin mixture, 1.0. The Vitamin mixture consisted of, in mg, thiamine·HCl, 5; riboflavin, 10; pyridoxine, 5; niacin, 20; calcium pantothenate, 25; inositol, 200; folic acid, 0.25; *p*-aminobenzoic acid, 2.5; biotin, 0.1. To this mixture, 99.73 g sucrose was added to make the total amount 100.00 g.

After feeding for 60 days, the body weight, red blood cell counts and hemoglobin content were estimated. Completely B_{12}-deficient animals indicated always lower values than those which had received vitamin B_{12}. The data are shown in Fig. 1 and Table I.

TABLE I

Erythrocyte Counts and Hemoglobin Content in B_{12}-Deficient Rats and in Control Animals

The Group received 0.2γ B_{12} per head daily				The completely B_{12}-deficient Group			
No.	Sex	Erythrocyte counts	Hemoglobin level	No.	Sex	Erythrocyte counts	Hemoglobin level
		$\times 10^6$	*g/100 ml*			$\times 10^6$	*g/100 ml*
1	♂	8.94	15.1	10	♂	8.08	12.0
2	♂	9.25	14.0	11	♂	7.89	11.6
3	♂	9.61	14.8	12	♂	7.20	11.2
4	♂	9.00	14.7	13	♂	6.39	11.2
5	♀	8.05	11.6	14	♀	8.00	9.2
6	♀	8.77	12.3	15	♀	6.32	7.9
7	♀	9.42	13.5	16	♀	7.09	9.8
8	♀	8.95	11.8	17	♀	6.56	8.8
9	♀	7.95	14.7	18	♀	5.06	9.1
				19	♀	6.48	8.1
Average		**8.88**	**13.6**	Average		**7.57**	**9.9**

TABLE II

The Change in Hemin-Fe^{55} and Nonhemin-Fe^{55} per ml of Blood after Administering Inorganic Fe^{55} of B_{12}-deficient Rats as Compared with Those Given B_{12}.

The Group given B_{12}					The completely B_{12}-deficient group				
No.	Hemin-Fe^{55}	Nonhemin-Fe^{55}	Hemin-Fe^{55} / Total Fe^{55}	Hemin-Fe^{55} per g Hb[a]	No.	Hemin-Fe^{55}	Nonhemin-Fe^{55}	Hemin-Fe^{55} / Total Fe^{55}	Hemin-Fe^{55} per g Hb
	cpm	*cpm*	*per cent*	*cpm*		*cpm*	*cpm*	*per cent*	*cpm*
1	91	13	87.5	603	10	61	11	84.7	508
2	68	0	100.0	486	11	48	16	75.0	414
3	101	8	92.7	682	12	21	0	100.0	188
4	125	14	89.9	850	13	41	14	74.5	366
5	73	16	82.0	629	14	21	7	75.0	228
6	66	5	93.0	528	15	21	11	68.6	304
7	121	14	89.6	896	16	49	5	90.7	500
8	115	15	88.5	975	17	15	4	77.2	170
9	125	10	92.8	850	18	33	10	76.7	363
					19	32	3	91.4	395
Average	98	**11**	**90.2**	**722**	Average	**35**	**8**	**81.4**	**344**

[a] Hemoglobin

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2. Formation of Fe^{55}-labeled Hemoglobin after Injecting Inorganic Fe^{55}.

The B_{12}-deficient rats indicating the low erythrocyte counts were used in the experiments. Each animal was injected intramuscularly (thigh muscle) 2 μc (8700 cpm) of inorganic Fe^{55}, and after 72 hours the change in Hemin-Fe^{55} and nonhemin-Fe^{55} levels was determined according to Brückmann *et al.* (6) with the results listed in Table II. The results indicated that the specific activity of Hemin-Fe^{55} per g of hemoglobin in B_{12}-deficient rats was one-half that of the group given B_{12}.

3. Preparation of Bone Marrow Homogenate.

The animal were killed and the bone marrow was quickly removed. Equal amounts of bone marrow from each rat from the same group were pooled, finely homogenized in the CESS medium, described below. The mixture was centrifuged for 15 min at 1500—2000 rpm, and purified as follows.

Bone marrow
↓ added cold CESS medium [a], agitated, and centrifuged for 15 min at 1500—2000 rpm.
Precipitate
↓ added cold CBSS medium, agitated, and centrifuged for 15 min at 1500—2000 rpm.
Precipitate
↓ filtered through a lint and homogenized for 5 min at 2000 rpm.
Bone marrow homogenate
↓
2.0 ml
↓ added solution A [b].
3.0 ml
↓
Shaking culture at 37°

[a] The CBSS medium is composed of, in g/*l*, sodium citrate ($Na_2C_6H_5O_7 \cdot 2H_2O$), 5.00; NaCl, 6.80; KCl, 0.37; $NaHCO_3$, 0.23; $CaCl_2$ (anhydrous), 0.17; $MgCl_2 \cdot 6H_2O$, 0.21; $Na_2HPO_3 \cdot 2H_2O$, 0.15; KH_2PO_4, 0.03; $MgSO_4 \cdot 7H_2O$, 0.07; glucose, 1.00.

[b] Solution A consists of 0.1 ml 0.2 *M* cysteine, 0.5 ml serum, solution of B_{12} and Fe^{55}, *etc.*

TABLE III

Increase in Hemoglobin upon Addition of Various Amounts of Vitamin B_{12} to Bone Marrow Homogenate of B_{12}-deficient Rat.

Amount of B_{12} added	Bone marrow homogenate of the rats given orally 0.2 γ B_{12} per head daily		Bone marrow homogenate of the completely B_{12}-deficient rate	
	Amount of hemoglobin	Increase in hemoglobin	Amount of hemoglobin	Increase in hemoglobin
mγ per tube	*g/100 g*	*per cent*	*g/100 g*	*per cent*
0	0.30	0	0.25	0
1	0.30	0	0.26	4.0
5	0.32	6.7	0.29	12.0
10	0.31	3.3	0.29	12.0
50	0.33	10.0	0.32	28.0
100	0.33	10.0	0.33	32.0
500	0.32	6.7	0.33	32.0
1000	0.32	6.7	0.32	28.0

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4. Effect of Incubation Time on the Increase of Hemoglobin Level.

The increase in hemoglobin formed following the addittion of B_{12} to bone marrow homogenate was tested for 60 min, and the maximum level of hemoglobin was observed at 30 min, as shown in Fig. 2.

Table III and Fig. 2. clearly show that the addition of vitamin B_{12} is effective for the formation of hemoglobin in bone marrow.

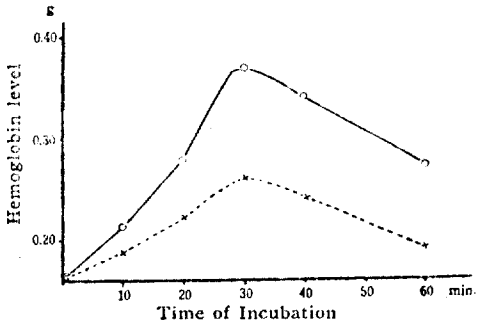

Fig. 2 *Increase in Hemoglobin in Bone Marrow Homogenate Supplemented with 0.1 γ of B_{12} per Tube of B_{12}-deficient Rats.*

o Increase in hemoglobin in bone marrow homogenate supplemented with 0.1 γ of B_{12} per tube of B_{12}-deficient rats.

× Negative control without supplementation of B_{12}.

5. Effect of Varying Amounts of Vitamin B_{12} on the Formation of Hemoglobin by Bone Marrow Homogenate.

To 2 ml of bone marrow homogenate were added varying amounts of vitamin B_{12}, and the increase of hemoglobin formed was studied by incubation for 30 min. The results are shown in Table III.

6. Synthesis of Fe^{55}-labeled Hemin from Inorganic Fe^{55} by Bone Marrow Homogenate of B_{12}-deficient Rats.

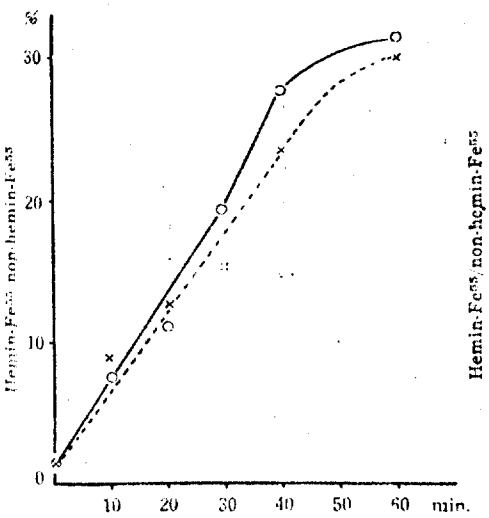

Fig. 3 *Contribution of Vitamin B_{12} to the Synthesis of Hemin-Fe^{55} from Inorganic Fe^{55} by Bone Marrow Homogenate of the Group Given B_{12} Daily.*

o Experiments of bone marrow homogenate supplemented with B_{12}

× Negative control without adding B_{12}

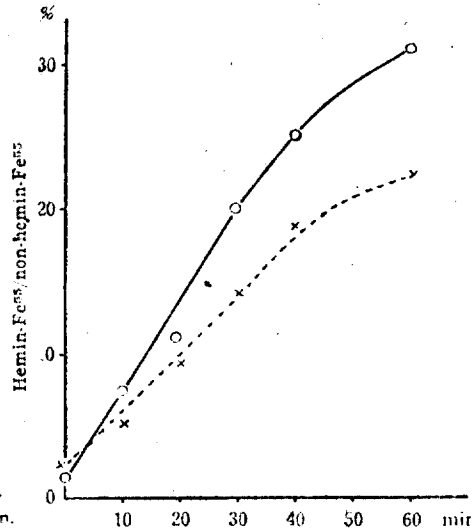

Fig. 4 *Contribution of Vitamin B_{12} to the Synthesis of Hemin-Fe^{55} from Inorganic Fe^{55} by Bone Marrow Homogenate of the Completely B_{12}-deficient Rats.*

o Experiments of bone marrow homogenate supplemented with B_{12}.

× Negative control without adding B_{12}.

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The final evidence was obtained for the synthesis of Fe^{55}-labeled hemoglobin by bone marrow homogenate of B_{12}-deficient rats, and the group supplemented with B_{12} was always found to produce more hemin-Fe^{55} than the negative control. Some examples are shown in Figs. 3 and 4.

SUMMARY

1. Inorganic Fe^{55} was injected to B_{12}-deficient rats, and the incorporation of inorganic Fe^{55} to hemin-Fe^{55} was confirmed.

2. The relation between vitamin B_{12} and the synthesis of hemin-Fe^{55} from inorganic Fe^{55} in bone marrow homogenate was studied, and the contribution of vitamin B_{12} to hemoglobin synthesis *in vitro* was established.

ACKNOWLEDGEMENT

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A simple assay of intrinsic factor-vitamin B_{12} complex employing the binding intrinsic factor antibody

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SYNOPSIS The reaction between binding intrinsic factor antibody and intrinsic factor-vitamin B_{12} complex has been studied. Initially in the zone of antibody excess, the relationship between the amount of antigen present and the amount of antigen-antibody complex adsorbed onto zirconium phosphate gel was linear. With increasing amounts of antigen, the curve departed from linearity and reached a plateau. The linear portion of the reaction forms the basis of a simple and reproducible assay for quantitating intrinsic factor to which vitamin B_{12} has already been bound. The assay provides a method for studying the fate of intrinsic factor-vitamin B_{12} complex during digestion and absorption. In two normal subjects given radioactive vitamin B_{12} orally, aspiration of ileal contents showed that only 50 to 70% of the radioactivity was bound to intrinsic factor at that level.

The study of the physiology of vitamin B_{12} absorption has been hampered by the lack of a simple assay for human intrinsic factor-vitamin B_{12} complex. The activity of human intrinsic factor-radioactive vitamin B_{12} complex can be measured *in vivo* in patients with pernicious anaemia (Heinle, Welch, Scharf, Meacham, and Prusoff, 1952; Schilling, 1953; Glass, Boyd, Gellin, and Stephanson, 1954; Baker and Mollin, 1955). However, this requires the cooperation of patients with pernicious anaemia, is time consuming, and comparatively insensitive. Human intrinsic factor-vitamin B_{12} complex can also be estimated *in vitro* by techniques employing ileal segments, everted ileal sacs, or small intestinal homogenates (Wilson and Wiseman, 1954; Wolff and Nabet, 1961; Sullivan, Herbert, and Castle, 1962; Boass and Wilson, 1963; Castro-Curel and Glass, 1963). These techniques have the disadvantage that they are specialized and difficult to apply to large numbers of samples.

The recognition of two types of intrinsic factor antibodies, the blocking antibody which prevents the combination of vitamin B_{12} with intrinsic factor, and the binding antibody which unites either with intrinsic factor or intrinsic factor-vitamin B_{12} complex (Garrido-Pinson, Turner, Crookston, Samloff, Miller, and Segal, 1966), has made possible the development of specific *in vitro* intrinsic factor assays.

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A variety of techniques employing the blocking antibody have been described, but these are only applicable to free intrinsic factor and cannot measure intrinsic factor already bound to vitamin B_{12} (Ardeman and Chanarin, 1963; Gottlieb, Lau, Wasserman, and Herbert, 1965; Hansen, Miller, and Tan, 1966). The binding antibody, because it combines with the intrinsic factor-vitamin B_{12} complex, is potentially capable of quantitating the latter. To date, the only ways of measuring intrinsic factor-vitamin B_{12} complex, employing the binding antibody, have been the electrophoretic retention method (Jeffries and Sleisenger, 1963; Bardhan, Wangel, and Callender, 1967) and the competitive inhibition of the antibody using sodium sulphate precipitation (Rothenberg, 1966). These are rather complicated techniques and do not appear to have gained wide acceptance. Moreover, in practice, these techniques have only been advocated as methods for measuring intrinsic factor activity, and their potential in quantitating the intrinsic factor-vitamin B_{12} complex has not been exploited.

This communication reports studies on the combination of the binding antibody with intrinsic factor-vitamin B_{12} complex and the use of this reaction in a simple and sensitive *in-vitro* assay for intrinsic factor-vitamin B_{12} complex. The assay has been employed in a preliminary study of the fate of intrinsic factor-vitamin B_{12} complex in the intestine.

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Materials and Methods

The technique is based on the fact that zirconium gel adsorbs protein complexes at or below their isoelectric point. The vitamin B_{12} binders in gastric juice, including intrinsic factor, are quantitatively adsorbed onto the gel at *p*H 5·0, but at *p*H above 5·5 the negative charge of the intrinsic factor and other gastric juice binders increases and they are not adsorbed onto the gel (Hansen *et al*, 1966). When the binding type antibody is attached to the intrinsic factor-vitamin B_{12} complex, the isoelectric point changes and it is adsorbed onto the gel at *p*H 6·25 (Jacob, Hansen, and Miller, 1966). Substances in solution not adsorbed onto the gel, including free radioactive vitamin B_{12}, can be removed by washing the gel with buffer.

Zirconium phosphate gels at *p*H 5·0 and *p*H 6·25 were prepared by the method of Hansen *et al* (1966). ^{57}Co-labelled radioactive vitamin B_{12} (specific activity 70 $\mu c/\mu g$) was obtained from the Radiochemical Centre, Amersham. This was diluted before use with non-radioactive vitamin B_{12} to give a specific activity of approximately 8 $\mu c/\mu g$. Radioactive measurements were carried out in a Packard automatic gamma scintillation counter, at least 10 000 counts being accumulated in each case.

Sera containing the binding and blocking antibody were obtained from four patients with pernicious anaemia. The titre of the antibodies in these sera was measured by zirconium gel assay (Hansen *et al*, 1966; Jacob *et al*, 1966). Control sera were obtained from healthy adults with no demonstrable intrinsic factor antibodies. To prevent radioactive vitamin B_{12} from binding to transcobalamin II in the serum and being adsorbed to the gel (Hansen, Tan, and Jacob, 1967), the vitamin B_{12} binding sites of the antibody sera and the control sera were saturated by adding excess non-radioactive vitamin B_{12} (5 ng/ml). When the sera were used as a source of blocking antibody, the unbound vitamin B_{12} was removed by albumin-coated charcoal prepared as described by Gottlieb *et al* (1965). The removal of excess unlabelled vitamin B_{12} was unnecessary when the serum was used as a source of binding antibody because the serum was added to the system after radioactive vitamin B_{12} was combined with intrinsic factor.

Gastric juices were obtained from healthy adult volunteers and patients with pernicious anaemia. The fasting juice was discarded. An intramuscular injection of pentagastrin (6 μg/kg body weight) was given and the juice aspirated at 15, 30, and 45 minutes thereafter. The juice was immediately filtered through glass wool, the *p*H adjusted to 10·0 with 1N NaOH to inactivate the pepsin and 30 minutes later brought back to 7·4 by the addition of 1N HCl. The juice was divided into aliquots and stored at −20 C until further use.

The intrinsic factor content of each sample of gastric juice was estimated by the use of the binding antibody in the assay described below and by the blocking antibody employing either albumin-coated charcoal (Gottlieb *et al*, 1965) or zirconium gel at *p*H 5·0 (Hansen *et al*, 1966).

Results

OBSERVATIONS ON THE COMBINATION OF BINDING ANTIBODY WITH INTRINSIC FACTOR-VITAMIN B_{12} COMPLEX

To determine whether the combination of the binding antibody with intrinsic factor-vitamin B_{12} complex could be used for assay of the complex, a detailed study of the reaction was undertaken using a fixed amount of antibody and increasing amounts of the complex. Radioactive vitamin B_{12} was added to gastric juice in excess of its total binding capacity to saturate all available intrinsic factor molecules. The juice was diluted 1 in 10 with 0·15M NaCl and duplicate amounts ranging from 0·05 to 1·0 ml were added to two sets of test tubes. To one set, 0·1 ml of serum containing 6·5 ng units of the binding antibody was added, and to the other set 0·1 ml of control serum was added. Ten ml of zirconium gel (*p*H 6·25) was then added to each tube. The tubes were mixed in a vortex mixer, centrifuged at 1000 *g* for five min and the supernatants discarded. The gel was washed three times with 15 ml of 0·1M am-

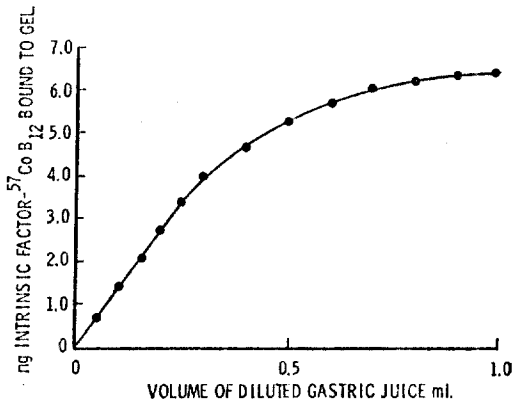

Fig. 1 *Relationship between volume of gastric juice containing excess ^{57}Co vitamin B_{12} and the amount, in ng units, of intrinsic factor-^{57}Co vitamin B_{12} bound to zirconium gel at pH 6·25. The gastric juice was diluted 1 in 10. The amount of antibody was kept constant throughout at 6·5 ng units.*

monium acetate buffer at *p*H 6·25. The radioactivity adsorbed onto the gel in the tube with antibody serum minus that in the corresponding tube with the control serum represented the amount of intrinsic factor-^{57}Co vitamin B_{12}-antibody complex adsorbed onto the gel. The results are shown in Figure 1. Initially, the relationship between the amount of gastric juice and the amount of intrinsic factor-^{57}Co vitamin B_{12}-antibody complex adsorbed onto the gel was linear up to about 3·4 ng units. As the amount of intrinsic factor-^{57}Co vitamin B_{12} complex was further increased, the amount of radioactivity bound to the gel for a given increment in intrinsic factor-vitamin B_{12} complex decreased. Finally a plateau was reached when the amount bound to the gel was equal to the antibody content of the serum. Increasing the intrinsic factor-vitamin B_{12} complex still further produced no change in the amount of radioactivity bound to the gel.

THE ASSAY

It has been found that the relationship between the amount of gastric juice saturated with ^{57}Co vitamin B_{12} used and the amount of intrinsic factor-^{57}Co vitamin B_{12} adsorbed onto the gel is always linear up to a value equivalent to approximately half the antibody content of the volume of serum employed. Using a volume of serum containing 5 ng units, the relationship is therefore linear to at least 2 ng units of intrinsic factor-^{57}Co vitamin B_{12} complex, although, at times, the linear range may extend further (Fig. 2). The protocol of a typical assay, for 1 volume of gastric juice to which excess ^{57}Co vitamin B_{12} has been added, is shown in Table I, and the value obtained from this, together with values obtained from other volumes of the gastric juice, is shown in Figure 2.

When this assay is used simply to quantitate intrinsic factor in gastric juice, a single dilution of gastric juice giving a value within the linear range is adequate. For gastric juices with a probable normal content of intrinsic factor 0·1 ml of a 1 in 10 dilution gives adequate results. Juices which are likely to contain less than 20 ng units/ml are assayed undiluted.

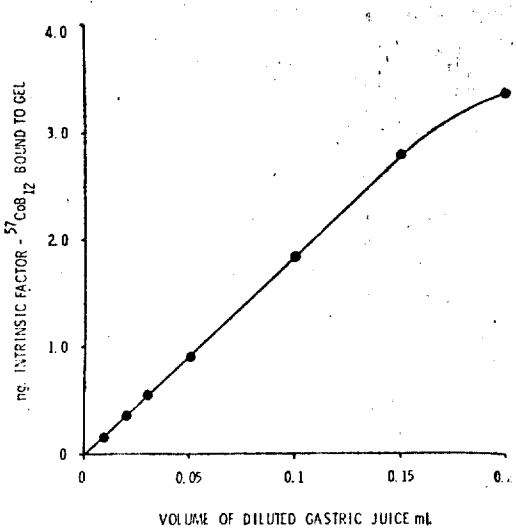

Fig. 2 *Assay of intrinsic factor-^{57}Co vitamin B_{12} complex of a normal gastric juice, diluted 1 in 10, to which an excess of radioactive vitamin B_{12} has been added. The amount of antibody serum employed contained 5 ng units of binding antibody. The graph is linear up to 2·8 ng units of intrinsic factor-^{57}Co vitamin B_{12}. The point at 0·01 ml of gastric juice is that referred to in the protocol in Table I.*

REPRODUCIBILITY

The intrinsic factor of one gastric juice (measured as intrinsic factor-^{57}Co vitamin B_{12} complex) was determined by this assay 12 times in one day. The values ranged from 158 to 163 ng units/ml with a coefficient of variation of 0·8% (Table II). Another gastric juice was stored in multiple aliquots and one of these was assayed in duplicate on eight different

0·15M NaCl (ml)	*Gastric Juice -^{57}CoB$_{12}$ Diluted 1 in 10 (ml)*	*Control Serum (ml)*	*Antibody Serum[a] (ml)*	*Zirconium Gel pH 6·25 (ml)*	*ng^{57}CoB$_{12}$ Bound to Gel*	*ng^{57}CoB$_{12}$ Bound by Binding Antibody[b]*
1·0	0·01	—	0·1	10	0·19	0·18
1·0	0·01	—	0·1	10	0·18	0·17
1·0	0·01	0·1	—	10	0·01	—
1·0	0·01	0·1	—	10	0·01	—

Table I *Assay protocol*[1]

[a]Only 1 volume of gastric juice-^{57}Co vitamin B_{12} is shown; the protocol for other volumes is identical.
[b]The antibody serum used contained 50 ng units of binding antibody per ml.
[c]ng units adsorbed onto gel using antibody serum minus the amount adsorbed using control serum. In practice, because of the high degree of reproducibility, duplicates may not be necessary.

days. The values ranged from 145 to 162 ng units/ml with a coefficient of variation of 3·8% (Table II).

Gastric Juice A		*Gastric Juice B*	
Date	*ng units/ml*	*Date*	*ng units/ml*
13-8-71	158	17-8-71	157
13-8-71	159		159
13-8-71	161	18-8-71	148
13-8-71	163		150
13-8-71	160	19-8-71	145
13-8-71	161		146
13-8-71	161	20-8-71	157
13-8-71	163		154
13-8-71	161	23-8-71	154
13-8-71	161		154
13-8-71	160	24-8-71	146
13-8-71	162		147
		27-8-71	162
			162
		31-8-71	158
			156

Table II *Reproducibility of the assay carried out on the same day (gastric juice A) and on eight different days (gastric juice B)*

COMPARISON WITH OTHER ASSAYS

The intrinsic factor content of 21 specimens of gastrict juice, as measured by the present assay for intrinsic factor-vitamin B_{12} complex, was compared with the results of intrinsic factor measurement using zirconium gel at *p*H 5·0 and the blocking antibody (Hansen *et al*, 1966). The results are shown in Figure 3. There is good correlation between the two assays (r = 0·99) although the binding antibody method gives a slightly lower value (approximately 5%) than the blocking antibody method.

The present assay was also compared with the albumin-coated charcoal assay (Gottlieb *et al*, 1965) (Fig. 4). Again there is good correlation between the results of the two assays (r = 0·99), although the binding antibody method gives values about 12% lower.

USE OF ASSAY IN MEASURING INTRINSIC FACTOR-^{57}CO VITAMIN B_{12} IN INTESTINAL JUICE

Two volunteers, with normal vitamin B_{12} absorption and normal secretion of intrinsic factor, were intubated orally with a fine radioopaque polyvinyl tube. When the distal end of the tube was in the upper ileum, a 1 μg dose of ^{57}Co vitamin B_{12} (1 μc) was given by mouth. Commencing 45 minutes later, ileal juice was aspirated at intervals with a syringe and the radioactivity of the aspirates monitored. The two specimens containing the most radioactivity were centrifuged at 4°C at 10 000 *g* for one hour and the supernatants removed. None of the sediments contained any significant amounts of radioactivity. The radioactivity in the supernatants was present in

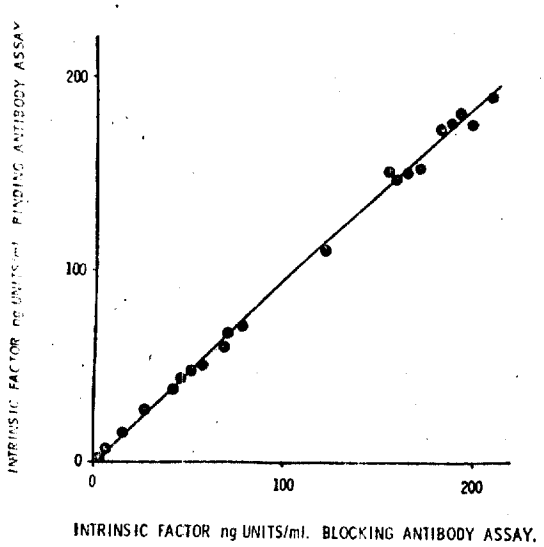

Fig. 3 *Intrinsic factor content of 21 specimens of gastric juice measured by the present assay (binding antibody assay) compared with the measurements by the blocking antibody zirconium gel assay (r = 0·99) (y = 0·96x − 0·71).*

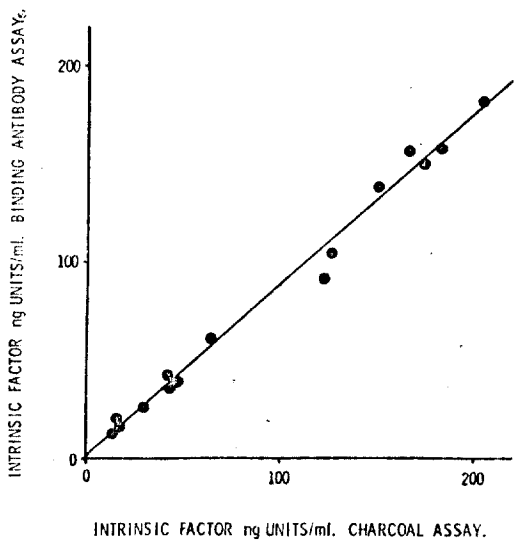

Fig. 4 *Intrinsic factor content of 15 specimens of gastric juice measured by the present assay (binding antibody assay) compared with the measurements by the blocking antibody charcoal assay (r = 0·99) (y = 0·87x + 0·76).*

	Time Specimen Obtained (hr after dose)	Total ng $^{57}CoB_{12}$/ml Aspirate	Unbound $^{57}CoB_{12}$ Removed by Albumin-coated Charcoal	Bound $^{57}CoB_{12}$	
				Intrinsic Factor-$^{57}CoB_{12}$ Complex	$^{57}CoB_{12}$ Attached to Non-Intrinsic Factor Binders
			% of Total	% of Total	% of Total
Patient A	1·5	9·8	18	52	30
	2·5	1·8	11	55	33
Patient B	1·0	1·6	19	69	12
	1·5	0·2	25	65	10

Table III *Results of study of vitamin B_{12} binding on ileal juice obtained after feeding radioactive vitamin B_{12}*

at least three forms: 'unbound' radioactivity which was measured and removed by treatment with albumin-coated charcoal; intrinsic factor-^{57}Co vitamin B_{12} complex which was measured by the present assay; and ^{57}Co vitamin B_{12} bound to unknown non-intrinsic factor binders, which was estimated by subtracting the other two quantities from the total amount of radioactivity in the supernatants. Table III shows the results of this study on two specimens of ileal juice from each of two patients and Fig. 5 the results of the intrinsic factor-vitamin B_{12} complex assay on one of the specimens.

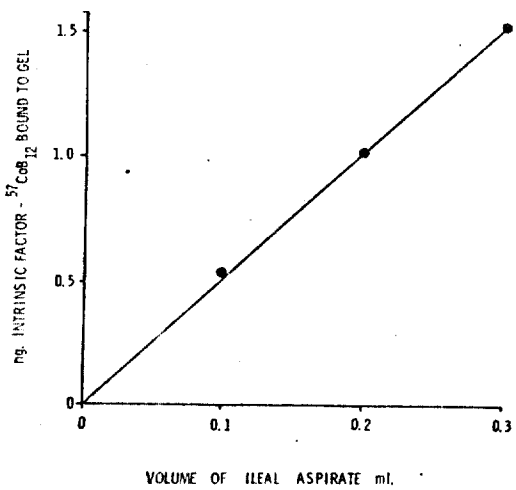

Fig. 5 *Assay of intrinsic factor-^{57}Co vitamin B_{12} complex aspirated from ileum of a normal subject 1·5 hours after an oral dose of 1 μg of ^{57}Co vitamin B_{12}. Serum containing 5 ng units of binding antibody was employed. 0·1 ml of aspirate contained 0·51 ng units of intrinsic factor-^{57}Co vitamin B_{12} complex.*

Discussion

A detailed study of the relationship between the binding antibody and intrinsic factor-vitamin B_{12} complex does not appear to have been previously undertaken. The results shown in Fig. 1 are typical of the combination of an antigen to an antibody which occurs in varying proportions. Using the initial part of the zone of antibody excess, the results given in this paper indicate that the reaction can be used to assay intrinsic factor-vitamin B_{12} complex. On a given day, the assay has a high degree of reproducibility. When carried out on different occasions, on aliquots of the same specimen, the variation is slightly greater. The reasons for this are not yet clear but the variations are not such as to interfere with the value of the assay.

The intrinsic factor content of gastric juices measured by the present assay tends to be a little lower than the results obtained using the two assays employing the blocking type antibody although there is an excellent correlation between the assays. Bardhan *et al* (1967) also noticed that the results of the charcoal assay using the blocking antibody were higher than their results using the binding antibody with the electrophoretic retention technique. Schade, Feick, Imrie, and Schilling (1967) have shown that, at room temperature, blocking antibody can displace some of the vitamin B_{12} already bound to intrinsic factor. It is possible that in the present assay the blocking antibody present in the binding antibody serum displaced some of the radioactive vitamin B_{12} from its combination with intrinsic factor, and so reduced the amount of radioactivity bound to the gel. This would result in a lower intrinsic factor measurement with the binding antibody. It is also perhaps not surprising that the binding and blocking assays give slightly different results, since they depend on two different antibodies, the one active at the site of vitamin B_{12} attachment to intrinsic factor and the other at some other site(s) on the intrinsic factor molecule. Of the two assays with the blocking antibody, the charcoal method gave the higher values. The explanation for this is not known. It was observed, however, that minor variations in the assay conditions, such as changing the brand of charcoal, or the amount of non-intrinsic factor

protein in the system, produced alterations in the estimation of total vitamin B_{12} binding and in the apparent intrinsic factor content when blocking antibody was added to the system. There is no evidence as to which of the assays gives a better measure of the physiological activity of intrinsic factor in promoting vitamin B_{12} absorption. Any of the three assays appears to be satisfactory for the estimation of intrinsic factor in routine clinical practice.

The present assay has been shown to be simple and reproducible and to give a good measure of intrinsic factor. In addition, it has the great advantage that it can be used to measure intrinsic factor to which radioactive vitamin B_{12} has already been bound. It is therefore capable of being used to quantitate accurately intrinsic factor-vitamin B_{12} complex such as is sometimes used in the diagnosis of pernicious anaemia and other vitamin B_{12} malabsorptive states.

The most important aspect of the present assay is its potential for the study of the physiology and pathology of vitamin B_{12} absorption. The preliminary results of the study of the fate of intrinsic factor-vitamin B_{12} complex in two subjects demonstrate this application of the assay. The findings are of interest in that only 50 to 70% of administered radioactivity recovered from the ileum was bound to intrinsic factor. This percentage was the same in each of the two specimens obtained from each patient, even though in patient A the second specimen was obtained one hour after the first. This may well explain why in normal subjects a test dose of radioactive vitamin B_{12} is never completely absorbed. Further studies are in progress.

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MEDICAL MEMORANDA

Sensitivity to Cyanocobalamin and Hydroxocobalamin

JEAN JAMES, ROBERT P. WARIN

British Medical Journal, 1971, 2, 262

The purpose of this paper is to draw attention to sensitivity to cyanocobalamin and hydroxocobalamin as a cause of chronic urticaria and to report the case of a patient who also reacted to vitamin B_{12} given by mouth.

Reports on sensitivity to cyanocobalamin are few, though sensitivity reactions to parenteral liver extracts are well recognized. Anaphylactic reactions to vitamin B_{12} injections, which in some cases were severe and included peripheral circulatory collapse, shivering, and death, have been recorded by Young *et al.* (1950), Borodulin (1961), Roy (1961), and Marinkovic (1962). Bedford (1952), Gillhespy (1955), and Ungley (1955) reported less severe allergic reactions. These findings were noted by Høvding (1968) in his report of a case of anaphylaxis after intramuscular injection of vitamin B_{12} in a patient who gave positive prick-test reactions to cyanocobalamin and hydroxocobalamin. Gillhespy (1955) reported that large doses of vitamin B_{12} can be tolerated by these patients when given by mouth.

Case Report

A woman aged 50 presented with symptoms of subacute combined degeneration of the cord in 1960 and was found to have pernicious anaemia, with a megaloblastic blood picture and a positive Schilling test. She was successfully treated with cyanocobalamin injections for the next eight years. In January 1968, however, she had two episodes of swelling of the face, shivering, bronchospasm, dyspnoea, and aphonia after injections of vitamin B_{12}, and developed persistent urticaria, which was treated with antihistamines.

In October 1968 she was admitted to hospital for investigation of the chronic urticaria. Her blood picture was normal. Intradermal and prick tests with aseptically diluted proprietary preparations of cyanocobalamin and hydroxocobalamin *B.P.* giving a concentration of 100 μg/ml resulted in a negative immediate allergic response, but a very small erythematous papule of doubtful significance was observed at this site five hours later. It was felt that her symptoms might be due to a "suggestion" effect of the vitamin B_{12} injections to which she attributed her symptoms; a change was therefore made, and cyanocobalamin cytamen 1,000 μg was given once monthly by intramuscular injection. The first injection given in hospital caused no symptoms, but after the second she again had an attack of shivering and bronchospasm, and her urticaria lesions were present for about four days in every week.

In January 1969 a further change was made and she was given Dictivit 100 μg by mouth twice daily (a vitamin B_{12} peptide, orally active and a fomented product of cyanocobalamin); again she had symptoms of bronchospasm, dyspnoea, flushing, dizziness, and headache, and her urticaria persisted. She then stopped all treatment herself for two months, with subsequent return of symptoms of subacute combined degeneration and peripheral neuritis. She was then given Anahaemin 2 ml intramuscularly each week (liver extract containing cyanocobalamin) and later Neo-Cytamen (hydroxocobalamin). Though she had no anaphylactic symptoms the urticaria was persistent and widespread in spite of treatment with antihistamines. Her treatment was then changed to Bifacton (25 mg of intrinsic factor plus vitamin B_{12} 7·5 μg = ½ *U.S.P.* anti-anaemia unit), but once more her urticaria persisted.

In November 1969 she was readmitted to hospital and found to have a normal blood count, but the pernicious anaemia was again confirmed by a Schilling test augmented with intrinsic factor. A glucose tolerance test was within normal limits and a barium-meal examination showed nothing abnormal apart from a small hiatus hernia. Her urticaria at this time was continuous and severe. She was treated with intrinsic factor 40 mg three times daily (supplied by Armour Pharmaceutical, Eastbourne), as Hogastrin had been out of production for several years, and her urticaria immediately subsided. In January 1970 she reported that she had not felt so well for years. Her serum vitamin B_{12} was 816 μg/ml (normal 150-925), but she reported some tingling of the fingers. *X*-ray examination of the cervical spine showed posterior disc lesion space narrowing of C 3/4, C 4/5, C 5/6, and osteophytes, but no narrowing of the intervertebral foramina. In February 1970 she had no symptoms of peripheral neuritis, anaphylaxis, or urticaria.

Comment

It appears that this patient was indeed sensitive to cyanocobalamin and hydroxocobalamin, responding with anaphylaxis and chronic severe urticaria. We cannot explain her negative response to intradermal prick tests with cyanocobalamin and hydroxocobalamin. She was unable to tolerate the drug administered by injection or by mouth, though Gillhespy (1955) reported that the latter route is tolerated by patients sensitive to the injection. At the time of writing the urticaria seemed to have cleared completely since taking intrinsic factor alone, and the pernicious anaemia appeared to be under control.

We would like to thank Mr. E. F. Wellington, chief pharmacist, Bristol General Hospital, for his help.

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Combined System Disease in Patients Treated with Oral Vitamin B_{12}

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Although the risk of damage to the nervous system occurring in patients with pernicious anemia treated with oral folic acid appears to be well recognized[1], the risk associated with the oral administration of vitamin B_{12} is in some danger of being forgotten. The following cases exemplify this.

Illustrative Cases

Case 1:

A 58 year old Caucasian woman had been diagnosed as suffering from pernicious anemia two and one half years prior to the present admission. This diagnosis was confirmed by finding histamine-fast achlorhydria and a megaloblastic bone marrow. She was treated at that time with six intramuscular injections of vitamin B_{12} and subsequently with 100-mg. tablets of vitamin B_{12} (Redisol: Merck, Sharp & Dohme) by mouth. These were at first taken at daily, and later at weekly, intervals. Her anemia was cured by this treatment, and she remained well until about three months before admission, when she began to experience feelings of numbness and occasional tingling in all four extremities, as well as a feeling of weakness in the legs. Five weeks before admission she noticed difficulty in walking, which worsened rapidly and was the reason for her referral.

Physical examination showed a white-haired woman with a marked degree of spastic rigidity in both lower limbs. Great force was necessary to bend them, and they showed the clasp-knife phenomenon. Her gait was stiff-legged and ataxic, and she required two canes in order to steady herself. Romberg's test was strongly positive. Sensory examination of the lower limbs revealed absent vibration sense and gross impairment of joint and position sense. Pin prick was appreciated normally, and light touch was normal apart from slight quantitative diminution over the toes. The knee jerks were hyperactive bilaterally and the ankle jerks hypoactive. Bilateral Babinski responses were present. The upper limbs showed normal tone, power, and coordination, as well as normal appreciation of all sensory modalities apart from minimal loss of light touch over the fingers of the right hand. Cranial nerves were unremarkable.

Laboratory findings included the following values: hematocrit, 44 per cent; mean corpusular volume, 125 cubic microns (normal, 82-92); mean corpuscular hemoglobin, 39 micro-micrograms (normal, 27-32); mean corpuscular hemoglobin concentration, 30 per cent (normal, 32-36), reticulocytes, 1.2 per cent, and hypersegmented polymorphonuclears. The bone marrow contained megoblasts, there was a histamine-fast achlorhydria, and the Schilling test was positive. The patient was found to be a mild diabetic.

Diagnoses of inadequately treated pernicious anemia with combined system disease and minimal polyneuropathy, and also mild diabetes mellitus were made. Treatment was undertaken with regular intramuscular injections of vitamin $B0_{12}$, physical therapy, and dietary control with oral tolbutamide for diabetes. The patient showed a moderate degree of improvement, so that by the end of three and one half months the slight loss of light touch was no longer present, the marked spasticity had largely disappeared, and she was able to walk with only one cane. She could then cross her legs, joint sense had improved, and Romberg's test was negative. The reflex changes, including the Babinski responses, and the loss of vibration sense persisted.

Case 2:

A 51 year old Caucasian woman was admitted to the hospital complaining of numb sensations in the feet, associated with difficulty in walking. By three or four days later these sensations had extended up to her waist, and within three weeks she had extreme difficulty with her gait, saying that she could not control her legs.

Physical examination showed a gray-haired woman who was unable to stand without support because of marked swaying, despite normal tone, power, and muscle bulk in all limbs, and normal deep and superficial reflexes. Sensory examination showed absent vibration sense extending up to the iliac spines, and partial loss of joint and position sense. Romberg's test exacerbated the instability on standing, and the heel-shin test showed a marked degree of ataxia.

Laboratory findings included the following: hematocrit 43 per cent, hemoglobin 13.7 Gm. per 100 ml., white blood cell count 7,500, with a normal differential. The bone marrow was normal. There was histamine-fast achlorhydria, and the Schilling test was positive.

Just prior to the onset of the illness the pa-

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tient had taken 64 pills at the rate of one a day, each containing 2 micrograms of vitamin B_{12} (Super Berlamins, Berlin), which was a sufficient quantity to explain the normal blood picture.

She was placed on regular intramuscular B_{12} therapy and showed marked improvement, so that by the end of one month her ataxia was less and appreciation of vibration was returning to the lower extremities. When seen one year later she had made further slight improvement, but her gait remained unsteady and Romberg's test was still positive.

Comment

Despite the known dangers of damage to the nervous system occurring when pernicious anemia is treated orally, patients continue to be seen who have acquired combined system disease while the anemia was apparently being controlled. Furthermore, the progression of weakness in untreated combined degeneration of the cord may be acute indeed, the patient sometimes regressing from a state of weakness to complete paraplegia within a week[2].

A great deal has been written during recent years suggesting that oral B_{12} in large doses may be adequate to control pernicious anemia[3]. However, Thompson and others[4] have shown that while daily 100-mg. doses of vitamin B_{12} taken by mouth enabled normal hematologic values to be maintained, the serum vitamin B_{12} levels fell progressively in every one of 37 patients tested. More recently it has been claimed that vitamin B_{12} peptide complexes are more effective[5], but this claim must be regarded as *sub judice* at the present time. Moreover, it has been shown repeatedly that the majority of patients are quite unreliable in taking oral medication with any regularity, and that spot checks on their urine and blood drug levels frequently fail to substantiate their protestations to the contrary. For these reasons, the intramuscular route, which is effective both in curing the anemia and in preventing nervous system damage, should continue to be used.

Summary

Two cases of combined system disease occurring in patients treated with oral vitamin B_{12} are described.

The oral route is condemned and reasons are given for continuing to administer the drug parentally.

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An *in vitro* Effect of Vitamin B_{12} on the Oxidation of Glutathione in Erythrocytes

By P. C. Jocelyn. (*Department of Biochemistry, University of Edinburgh*)

When washed erythrocytes are oxygenated, the rate of oxidation of glutathione in the cells depends on the constitution of the suspending medium. Using saline or human dialysed serum or plasma (HDP) as the medium, the rate of oxidation of glutathione is increased by Cu^{2+} (50–200 μg. %), SeO_2 (100–400 μg. %) and ascorbic acid (100 mg. %).

When human dialysed serum or plasma is obtained by a short dialysis (e.g. 24 hr.) against copper-free saline, the rate of oxidation of glutathione in the cells is often inhibited by vitamin B_{12} added at concentrations of 100–10 000 $\mu\mu$g./ml. of medium. If this inhibition is considerable, Cu^{2+} (25–100 μg. %) subsequently added, neutralizes the effect of the vitamin. On the other hand, when the inhibition is small, the increase in rate of oxidation of glutathione induced by Cu^{2+} is neutralized by the vitamin. Addition of vitamin B_{12} to haemolysates does not produce any inhibition.

The results suggest that the vitamin may inhibit the entry of Cu^{2+} into erythrocytes.

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VITAMIN B_{12} INTRINSIC FACTOR, Symp., HAMBURG

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Vitamin B_{12} in Carbohydrate Metabolism

By B. C. Johnson, D. K. Biswas, S. R. Wagle and S. Gurnani

It has been reported that the incorporation of serine-2-C^{14} and of glucose-U-C^{14} into glycogen is depressed in the vitamin B_{12}-deficient animal (Johnson, Am. J. Clin. Nutrition, 1958, *6*, 34). This effect of vitamin B_{12} deficiency has recently been reexamined, with the finding of lowered incorporation of radiactivity from labeled glucose in particular, and also from serine and alanine. At the same time, there was no effect on protein labeling from serine or alanine, although there was from glucose. In contrast to the situation in *Ochromonas* (Arnstein and White, Biochim. Biophys. Acta, 1959, *36*, 286), no effect of B_{12} deficiency on serine biosynthesis from glucose was found.

The effect of vitamin B_{12} deficiency on the incorporation of radioactivity from labeled glucose into glycogen has been studied *in vitro*, since this appeared to be one of the most striking effects observed *in vivo*. Using whole liver homogenate plus the cofactors CoA, UTP, aspartate, and Mg^{++}, it was found that the incorporation of C^{14} from glucose into glycogen is reduced by approximately one-half in livers from vitamin B_{12}-deficient animals. The effect of addition DMBC and of some purification of the enzyme system will be reported.

In other work, using the DR-aldolase system of Boxer *et al.* (J. Biol. Chem., 1958, *233*, 535), it has been found that vitamin B_{12} deficiency has no effect on deoxyribose biosynthesis by $C_2 + C_3$ condensation.

National Institute for Medical Research, London

The Function of Vitamin B_{12} in Amino Acid and Protein Biosynthesis by Ochromonas malhamensis

By H. R. V. Arnstein and A. M. White

Cell suspensions of *O. malhamensis* incorporate more labelled phenylalanine or glucose into protein when cyanocobalamin is added. The percentage increase in the incorporation due to vitamin

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Natl. Vitamin Foundation, Nutrition Symposium Ser No 7, 119-32 (1953)

VITAMIN B_{12} AS A GROWTH FACTOR IN ITALIAN CHILDREN ON DIETS LOW IN ANIMAL PROTEIN*

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GIORGIO MAGGIONI, SALVATORE CORBO AND
GIUSEPPE LANCIANO

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Since 1945, we in the nutrition clinics have had as one of our major problems that of "simple growth retardation" in children. By the term "simple growth retardation" we mean underweight in children due to a calorie debt, which may or may not include a protein debt, not caused by organic disease, endocrinopathies and deficiencies of presently recognized essential nutrients. The recognition and treatment of children with these conditions are either well known or not of immediate concern to this study. On the other hand the treatment of "simple growth retardation" is of considerable concern to parents, school authorities and to many professional people. This is true even though we are not always sure that "simple growth retardation" represents necessarily a harmful condition other than the psychological handicap imposed by being different from the majority.

With these factors in mind a two year study was undertaken on 200 children with "simple growth retardation" between the ages of 7 and 11 years who were all subjected to intensive dietary education and all were given supplementary amounts of vitamins A and D. One-half of the children belonged to families whose food supply was sup-

* Supported by a grant-in-aid from The National Vitamin Foundation, Incorporated.

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plemented by one-half pound of enriched white bread daily for each member of the family. Equally good weight gains were obtained in both groups with about half of the children reaching a weight for height sufficient to be considered normal. A second study was then undertaken in which one group received a multiple vitamin capsule containing supplementary amounts of vitamins A, D, and C, and thiamine, niacinamide and riboflavin. The control group received only supplementary amounts of vitamins A and D. Again the weight gains over the next two years were similar in the two groups.

It was at this time (1949) that vitamin B_{12} became available for oral administration. Also there appeared the preliminary paper of Wetzel, Fargo, Smith and Helikon[1] who reported positive effects as demonstrated by the Wetzel Grid Technique in 5 of 11 children in "simple growth failure." It seemed to us that vitamin B_{12} might influence our children with "simple growth retardation" who had failed to respond by dietary education and to vitamin supplementation with the presently recognized "essential" vitamins. Even though we have had changes in the channel of the Wetzel grid we have not been able to determine how many represent responses to vitamin B_{12} or how many represent chance or a delayed response to dietary education.

It was at this point in the spring of 1951 that Dr. Gino Frontali, Professor of Pediatrics at the University of Rome and Director of the National Center for the Study of Children's Alimentation in Italy, offered through Dr. Roberto Funaro to make a cooperative study with us on the effects of vitamin B_{12} on underweight children on low protein diets in Italy. The National Vitamin Foundation agreed to support this study if final and definitive arrangements could be made and Merck and Company agreed to

furnish the vitamin B_{12} tablets and placebos. Accordingly, Dr. Funaro went to Italy and during the summer and fall of 1951 together with Professor Frontali and his group, outlined and planned the details of the study and selected an orphanage in Rome, the Asilo Savoia, and an elementary school in Gaeta as the site of the study. The observations throughout the school year were made by the Italian group and then tabulated and analyzed by both groups in the summer and fall of 1952.

The period of observation and administration of the tablets of vitamin B_{12} and the placebos was fixed from November to June. This plan has allowed us to follow for a period of 7 months two groups of children: (a) boys and girls 6 to 10 years of age living in an orphanage on a diet of known composition for the group, and (b) boys and girls 6 to 12 years of age living at home, of various economic categories, but in a depressed area, where the diets were known to be poor. This was checked by diet history obtained on each subject twice during the 7-month period.

All the children at the beginning of the study were subjected to a thorough medical examination with special emphasis on signs of deficiency diseases and which included a tuberculin patch test, X-ray of the chest in the Asilo Savoia, and fluoroscopic examination of the chest in Gaeta. The heights, weights, and chest measurements were made by one of us, the heights and weights at monthly intervals, and the chest measurements in November, March, and May. This has permitted recording of the results on the Correnti Auxogram[2] in November, March, and May. In addition records of absenteeism, illnesses and academic progress were kept. At both locations the children were grouped by class, age, and sex into two homogeneous groups.

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To one group were administered 20 micrograms of vitamin B_{12}. To the other were given the placebo tablets identical in appearance to the B_{12} tablets. At the Asilo Savoia the tablets were administered daily except Sunday by the sisters. At the elementary school the tablets were administered on all 6 school days, holidays excepted, by two sanitary assistants. Neither the sisters nor the sanitary assistants knew that one of the tablets was a placebo but they did know that we were testing which of the tablets was better.

Results

At the Asilo Savoia

Here direct control over the food, the central kitchen, and the common table allowed a group dietary analysis. In Table I are given the daily averages for a typical week compared with Frontali-Bacchetta findings in post-war Central Italy and with the U. S. National Research Council's Recommended Daily Allowances for children 7 to 12 years of age. The average total daily calories of 1826 is 20 per cent below the Recommended Daily Allowances and 10 per cent below the average value for children in Central Italy. It is more comparable with the 1775 average daily calories of children of the white collar workers in Italy. It is this group in Italy that has the lowest calorie intake.

Table I

Calorie and Protein Values in the Diet of Children of the Asilo Savoia

In Comparison with the Italian Children's Diet and with the R.D.A.

	Asilo Savoia	Average Central Italy	R.D.A.
Total Calories	1825	2033	2250
Total Protein	69 gm.	79.2 gm.	65 gm.
Animal Protein	27 gm.	29.9 gm.	32.5 gm.

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The total protein intake of 69 grams is considerably below the 79 grams consumed by children in Central Italy but it is not significantly different from the average of the Recommended Daily Allowance of 65 grams for children 7-12 years of age. The amount of animal protein however is only 27 grams as compared to about 30 grams in Central Italy and the 32.5 grams of the Recommended Daily Allowances.

It thus seems that the diet in the Asilo Savoia is definitely less in calories and in animal protein than the Recommended Daily Allowances and about the same as the lowest economic group in Central Italy. The total protein intake however equals the Recommended Daily Allowances.

Changes in Weight

At the end of the period of observation in June, 1952, with the unavoidable losses due to illness or to transfer of certain children to other institutions or to foster homes there remained a total of 159 under observation, divided as follows: boys receiving vitamin B_{12}—44; boys receiving placebos—44; girls receiving vitamin B_{12}—37; girls receiving placebos—34. Tables II and III indicate that the two groups of boys and girls were relatively homogeneous in age distribution. They were also homogeneous as to initial weights. The average initial weight of the treated boys was 23.2 Kg. ± 3.23 against 23.8 Kg. ± 3.22 in the controls. The average initial weight of the vitamin B_{12} treated girls was 24.9 Kg. ± 4.89 against 24.9 Kg. ± 4.36 in the controls. Also in Tables II, III and IV are given the average weights for age groups in November and June along with the absolute gain in weight. Both boys and girls, treated and control alike, gained weight over the previous November, but in every age group for both boys and girls the gain in weight for the vitamin B_{12}

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group was greater than in the placebo treated group. This fact in itself is very significant in spite of the fact that there are too few in each age group to be statistically significant. The entire group of boys treated with vitamin B_{12} gained on the average 341 grams more than did the placebo treated boys. The entire group of vitamin B_{12} treated girls gained on the average 365 grams more than did the placebo treated girls. The statistical significance of these two groups is indicated by a p slightly greater than 0.05 in the girls and a p slightly less than 0.05 in the boys.

Table II

Asilo Savoia—Boys

Age Years	Number of Treated Children	Average Weight In November	Average Weight In June	Gain in Weight in 7 Months
Treated with vitamin B_{12} (Initial Wt. 23.2 ± 3.23).				
6	2	19.100	20.150	+1050
7	18	21.288	22.915	+1627
8	11	24.672	26.435	+1763
9–10	13	25.546	27.353	+1807
Total	44	Average Total Gain		1688
Controls (Initial Weight 23.8 ± 3.22).				
6	4	19.850	20.600	+ 800
7	15	21.700	22.953	+1253
8	13	25.069	26.669	+1600
9–10	12	26.508	27.883	+1365
Total	44	Average Total Gain		1347

Table III

Asilo Savoia—Girls

Age Years	Number of Treated Children	Average Weight In November	Average Weight In June	Gain in Weight in 7 Months
Treated with vitamin B_{12} (Initial Weight 24.9 ± 4.898).				
6	6	18.700	20.300	+1600
7	8	23.187	24.824	+1637
8	8	24.150	25.587	+1437
9	7	26.442	28.399	+1957
10	8	30.737	33.362	+2625
Total	37	Average Total Gain		1862
Controls (Initial Weight 24.9 ± 4.361).				
6	8	20.130	21.455	+1325
7	3	22.300	22.966	+ 666
8	7	24.180	25.508	+1328
9	7	25.371	26.713	+1342
10	9	30.133	32.310	+2177
Total	34	Average Total Gain		1497

Table IV

Asilo Savoia

Difference of Gain in the Treated Group In Comparison with the Control Group

BOYS

Age	
6	+250
7	+374
8	+163
9-10	+442
Total Average Gain	+341

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GIRLS

Age	
6	+275
7	+973
8	+109
9	+615
10	+448
Total Average Gain	+365

Changes in Height

The absolute and percentage increases in height and changes in the Correnti Auxogram for the boys and girls in both vitamin B_{12} and placebo groups, age by age, has been computed and found not to be indicative of any differences.

At the Gaeta Elementary School

The dietary study of the school children who are the subjects of this study will be reported upon in detail at a later time by Dr. Corbo. In Table V are compared the average daily intake of our subjects, male and female, with the Frontali-Bacchetta findings in post-war Southern Italy and with the National Research Council's Recommended Daily Allowances. The total calories of the boys are almost identical to the average in Southern Italy and about 20 per cent lower than the Recommended Daily Allowances. The girls however are 30 per cent lower than the Recommended Daily Allowances and about 10 per cent lower than the average for Southern Italy. Total proteins are significantly lower in both males and females than the average for Southern Italy and somewhat lower than the Recommended Daily Allowances. This difference is greater in

the females than in the males. The intake of animal protein in both boys and girls is clearly inferior to that of the Recommended Daily Allowances being about half in the boys and about two-thirds in the girls.

Table V

Calorie and Protein Values in the Diet of Children of the School of Gaeta

In Comparison with the Average Italian Children's Diet and with the R.D.A.

	School of Gaeta Boys	School of Gaeta Girls	Average Southern Italy	R.D.A.
Total Calories	1772	1573	1764	2250
Total Proteins	62.08 gm.	59.55 gm.	72.90 gm.	65 gm.
Animal Proteins	17.32 gm.	24.25 gm.	22.40 gm.	32.5 gm.

Changes in Weight

At the end of the period of observation in June, 1952 there remained a total of 199 children under observation as follows: boys receiving vitamin B_{12}—52; boys receiving placebos—58; girls receiving vitamin B_{12}—37; girls receiving placebos—52. Tables VI and VII indicate that the age grouping, particularly for the girls, is not as homogeneous as at the Asilo Savoia. In both boys and girls there were more control subjects than treated subjects. The average initial weights of the vitamin B_{12} treated boys were 28.5 Kg. ± 4.64 against 26.9 Kg. ± 3.81 in the controls. The average initial weights of the vitamin B_{12} treated girls were 24.5 Kg. ± 3.24 against 24.8 Kg. ± 3.08 in the controls. Thus the homogenity of the groups in the Gaeta elementary school was not as great as in the Asilo Savoia. In Tables VI, VII and VIII are given the average weights for age in November and June along with the absolute gain in weights. As in the Asilo Savoia, both boys and girls,

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treated and control alike, gained weight in June over the preceding November. In the boys the vitamin B_{12} group gained more weight in each age group than did the control group except for the 9-year olds where a 57 grams difference in favor of the control group was noted. In the girls the vitamin B_{12} treated group gained more weight at every age except the 7 and 8 year olds where the numbers in these two age categories are very dissimilar. Statistically none of the results is significant for each age-sex group as the numbers are too small. The entire group of boys in Gaeta treated with B_{12} gained 497 grams more than the control group of boys. This difference has a p slightly less than 0.05. The entire group of girls in Gaeta treated with vitamin B_{12} gained 219 grams more than the control girls. This difference has a p greater than 0.05.

Table VI

School of Gaeta—Boys

Age Years	Number of Treated Children	Average Weight In November	Average Weight In June	Gain in Weight in 7 Months
Treated with vitamin B_{12} (Initial Wt. 28.5 ± 4.643).				
8	15	26.213	28.179	+1967
9	14	27.650	28.992	+1343
10	12	29.383	30.700	+1317
11	8	33.337	35.662	+2325
12	3	28.033	29.700	+1667
Total	52	Average Total Gain		+1677
Controls (Initial Weight 26.9 ± 3.81).				
8	20	25.295	26.445	+1150
9	19	26.742	28.142	+1400
10	10	28.110	28.150	+1040
11	6	26.825	27.752	+ 967
12	3	33.210	33.967	+ 757
Total	58	Average Total Gain		+1180

Table VII

School of Gaeta—Girls

Age Years	Number of Treated Children	Average Weight In November	Average Weight In June	Gain in Weight in 7 Months
Treated with vitamin B_{12} (Initial weight 24.5 ± 3.238).				
7	13	22.784	24.214	+1430
8	3	20.666	21.976	+1300
9	12	25.825	27.558	+1733
10	7	25.414	27.157	+1743
11	2	30.700	32.100	+1400
Total	37	Average Total Gain		+1575
Controls (Initial weight 24.8 ± 3.082).				
7	4	23.675	25.225	+1500
8	11	22.963	24.399	+1436
9	16	24.981	26.224	+1243
10	17	25.155	26.558	+1403
11	4	28.450	29.400	+ 950
Total	52	Average Total Gain		+1356

Table VIII

Difference of Gain in the Treated Group In Comparison with the Control Group

School of Gaeta

BOYS

Age	
8	+817
9	— 57
10	+277
11	+1358
12	+910
Total Average Gain	+497

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GIRLS

Age	
7	— 70
8	—136
9	+490
10	+340
11	+450
Total Average Gain	+219

Changes in Height

As in the Asilo Savoia the absolute and percentage increase in height and changes in the Correnti Auxogram for the boys and girls in both the vitamin B_{12} and control groups, age by age, has been computed and found not to be indicative of any difference.

Combined Results of Asilo Savoia and the Elementary School at Gaeta

Since both boys and girls at both localities receiving vitamin B_{12} made a greater gain in weight than did their companions receiving placebo tablets, and since in 2 of these groups the degree of significance equalled a p greater than 0.05 and in 2 groups the p was slightly less than 0.05 it seemed likely that had we larger numbers in each of these groups the results would be statistically significant. Since we included children in comparable age groups, boys 6 to 12, and girls 6 to 11, in which sex plays no particular role as far as weight for age and height is concerned and since in both groups the pre-puberty spurt is largely eliminated we feel it is justified to combine the boys and girls at both Asilo Savoia and at Gaeta into two groups, those treated with B_{12} and those given placebos. When this is done by

the method of covariance analysis of the combined data the results indicate that the greater gain in weight in June of the vitamin B_{12} treated group had a p of 0.001. Hence the greater gain in weight in favor of the B_{12} treated children may be considered highly significant.

Summary

A group of 351 children consisting of boys between the ages of 6 and 12 and girls between the ages of 6 and 11 residing either in an orphanage (Asilo Savoia) in Rome or living in a depressed area and attending an elementary school at Gaeta who were subsisting on a diet containing 20 to 30 per cent less calories than the Recommended Daily Allowances and 15 to 40 per cent less animal protein than the Recommended Daily Allowances were divided into two approximately similar groups one of whom received 20 micrograms of vitamin B_{12} daily by mouth and the other a placebo tablet of similar appearance. The 172 who received vitamin B_{12} made an average gain of 345 grams more than the group of 179 who received the placebo tablets. This increased gain in weight is highly significant with a probability of it occurring by chance being only 1 in 1000. When these results are divided into boys and girls residing at the Asilo Savoia or in Gaeta and compared with the same groups receiving placebos, each group receiving B_{12} made better weight gains than the placebo group. In 2 of the groups the results are of low order of significance and in 2 groups they probably are not significant. Further subdivision into age groups are statistically not significant.

We found no differences in gains in heights in the two groups.

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The period of observation, 7 months, was too short for valid analysis of the data by Correnti's Auxogram. Observations are being continued for an additional year.

We conclude that under the conditions of our study the daily oral administration of 20 micrograms of vitamin B_{12} resulted in a highly significant increase in weight gain for a period of 7 months in children between the ages of 7 and 11 whose diets were lower in calories and in animal protein than that of the Recommended Daily Allowances.

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Gut, 1963, 4, 231

The vitamin B12 content of human liver tissue obtained by aspiration biopsy

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EDITORIAL SYNOPSIS It is possible to estimate the vitamin B12 content of liver specimens obtained by needle biopsy. The liver B12 content is not related to the serum levels of vitamin B12, bilirubin, alkaline phosphatase, glutamic-oxaloacetic transaminase, or albumin. It is reduced in a number of pathological conditions of the liver and the reasons for this are discussed, as also are the factors determining the content of B12 in the normal liver.

Vitamin B12 (cyanocobalamin) is normally absorbed, probably by the intrinsic factor mechanism in the lower ileum. It is stored in the liver and from there transported to the bone marrow and other sites for utilization in nuclear maturation. It is excreted in the urine, and to a lesser extent, in the bile (Stokes, 1961).

Because of difficulty in obtaining human liver for analysis, studies of vitamin B12 storage and deficiency in man have perforce been made chiefly by estimation of serum B12 levels. These studies have been reviewed by Cowling and Mackay (1959) and by Smith (1960), among others.

The serum vitamin B12 level in health normally lies between 160 and 900 $\mu\mu$g. per ml., being low in B12 deficiency and raised following parenteral B12 therapy. In both experimental liver injury and clinical liver disease high serum B12 values are not infrequent (Stein, Stein, Aronovitch, Grossowicz, and Rachmilewitz, 1956; Cowling and Mackay, 1959). They are most often found in diffuse parenchymal cell damage and neoplastic involvement of the liver, and occasionally present in nutritional cirrhosis and active chronic hepatitis. Rarely, the rise may be extreme, as in the patient with 'obstructive hepatic necrosis' reported by Mackay, Cowling, and Gray (1957), and in some patients with metastatic carcinoma of the liver.

Several factors have been implicated in these changes, but release of stored vitamin B12 from damaged parenchymal cells appears quantitatively most important. There may also be a decrease in hepatic B12 uptake from portal blood (Glass, 1959), impaired storage of B12 by damaged liver cells, and possibly changes in the B12-binding capacity of serum, although increased α-globulins which bind B12 (Pitney, Beard, and Van Loon 1954) are not usual in liver disease.

Direct studies of liver B12 content have not been extensive and have been made largely on necropsy material. The reported results vary slightly, depending on methods of extraction and assay of B12. Ross and Mollin (1957) and Pitney and Onesti (1961) both reported higher B12 yields when assay followed papain digestion. Swendseid, Hvolboll, Schick, and Halsted (1957) reported a mean value of 0·70 μg. per g. wet liver tissue in 132 necropsy specimens, using *L. leishmannii* as test organism, while Pitney and Onesti (1961) found a mean value of 0·78 μg. per gram with a range from 0·61 to 1·02 μg. per gram wet liver tissue. They used *Euglena gracilis* as test organism after preliminary papain digestion. In disease, Girdwood (1952) found negligible amounts of vitamin B12 in the liver of a patient dying with untreated pernicious anaemia. Swendseid and her colleagues (1957) reported a low mean level of 0·26 μg. per g. in necropsy specimens from cirrhotic patients.

Studies of the vitamin B12 content of tissue obtained by needle biopsy of the liver have appeared in recent years. French workers (Wolff, Drouet, and Karlin-Weissman, 1951) first developed this technique, and demonstrated low liver B12 levels in pernicious anaemia with restoration towards normal following treatment with vitamin B12. In succeeding years, however, few reports dealing with this subject appeared in the literature, possibly because of doubts of the accuracy of an assay necessarily made on a small amount of fragmented tissue. This problem has been resolved by the work of Jhala and Gadgil (1960) and of Pitney and Onesti (1961).

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Both groups estimated vitamin B12 activity by modifications of the method of Ross (1952), and compared the B12 content of specimens obtained by biopsy needle with those of tissue blocks obtained at the same time at necropsy. Jhala and Gadgil (1960) found close agreement between the two in 10 of 12 instances. Their 'biopsy' weights ranged from 4·2 to 10 mg. Discordant results occurred with weights of 5 and 7 mg. Pitney and Onesti (1961), in a more extensive investigation of both normal and cirrhotic livers, found that tissue blocks from either lobe had a similar B12 content, and that the assay of the biopsy agreed with assay of the block to within 20%, provided the biopsy weight was at least 7 mg. With lesser biopsy weights there were larger discrepancies. It is probable, therefore, that assay of liver biopsy specimens gives a satisfactory estimation of liver B12 content.

The only extensive investigations of liver B12 content using liver biopsy have been those of Nelson and Doctor (1958, 1960, 1962). They used *L. leishmannii* as test organism without preliminary papain digestion, but reported a higher normal range, from 1·41 to 2·58 (mean 1·94) μg. per g. wet liver tissue. Values were low in cirrhosis, fatty liver, obstructive jaundice, and viral hepatitis. In neoplastic disease results varied, the liver B12 content being lowest in specimens containing metastatic tumours. They also noted elevations of serum B12 levels in all types of malignant neoplasm, the mechanism of which was uncertain.

The present paper is an extension of these results, especially of those of Pitney and Onesti (1961), and reports changes in liver B12 content of biopsy specimens in relation to clinical, histological, and biochemical changes in 68 patients with various diseases.

PATIENTS STUDIED AND LABORATORY METHODS

Liver tissue for bio-assay was obtained on 70 occasions from 68 patients, comprising 44 males and 24 females. Their ages ranged from 19 to 81 years with a mean of 54 years. The majority (49) were between 40 and 70 years of age. All were of Caucasian stock except four Australian aboriginals and three of mixed race. None had received vitamin B12 parenterally before biopsy. The indications for liver biopsy were the differential diagnosis of jaundice, hepatomegaly, or disseminated granulomatous disease. The wet weight of liver tissue available for assay ranged from 7·0 to 28·7 mg. with a mean of 12·6 mg.

Vitamin B12 assay was performed after papain digestion using the Z strain of *Euglena gracilis* (Pitney and Onesti, 1961). The normal values for this method lie between 0·61 and 1·02 μg. per g. wet liver tissue. Serum B12 estimations followed the method of Ross (1952), the normal range for this laboratory being from 160 to 900 $\mu\mu$g. per ml. (Nicholas and Pitney, 1958). Histological and biochemical estimations were performed by standard methods.

RESULTS

Allowing for differences in diagnosis, the results were not affected by the age or sex of the patient, or the month of the year in which biopsy was performed. Biopsies from aboriginal patients were too few to allow assessment of possible racial factors. The mean B12 content of biopsies from female patients was slightly lower than that of biopsies from male patients, 0·48 μg. per g., compared with 0·59 μg. per g., but the difference was of doubtful statistical significance ($0{\cdot}1 > p > 0{\cdot}05$).

The relation between histological diagnosis and liver B12 content is summarized in Table I, and individual results in each group are shown in detail in Figure 1.

TABLE I

VITAMIN B12 CONTENT OF LIVER BIOPSY SPECIMENS IN RELATION TO HISTOLOGICAL DIAGNOSIS

Histological Diagnosis	*No. of Cases*	*B12 Content (μg./g.)*	
		Mean	*Range*
Normal histology	9	0·87	0·39-1·33
Fatty change	12	0·47	0·19-0·93
Parenchymal cell damage	10	0·64	0·25-0·93
Metastatic neoplasm	9	0·47	0·20-0·75
Portal cirrhosis	7	0·43	0·19-0·89
Cholestatic jaundice	7	0·55	0·26-1·17
Viral hepatitis	5	0·57	0·29-1·11
Haemochromatosis	5	0·49	0·18-0·73
External biliary obstruction	4	0·25	0·16-0·45
Dubin-Johnson syndrome	1	0·92	—
Multiple granulomata	1	0·72	—

The biopsy was histologically normal in 12 cases, but in three later examination disclosed extensive metastatic carcinoma in the liver and these have, therefore, been excluded. The mean and range of the other nine specimens agree closely with those of Pitney and Onesti (1961), providing further evidence of the validity of bio-assay of small specimens. There was only one low result, 0·39 μg. per g. in a man of 48 years with megaloblastic anaemia due to nutritional folic acid deficiency. Since his serum B12 was at the lower normal limit, 160 $\mu\mu$g. per ml., it is probable that his diet was also deficient in vitamin B12.

In 12 biopsies the chief histological change was fatty metamorphosis, and the B12 content was below normal in 10 of these with a mean of 0·47 μg. per g. In eight of the 12 cases the fatty change was associated with alcoholism and the mean B12 content of these with 0·39 μg. per g. It is possible that two factors are involved, replacement of parenchymal tissue by lipid and inadequate B12 intake due to malnutrition in chronic alcoholics.

Ten biopsies showed only slight to moderate non-specific parenchymal cell damage without gross

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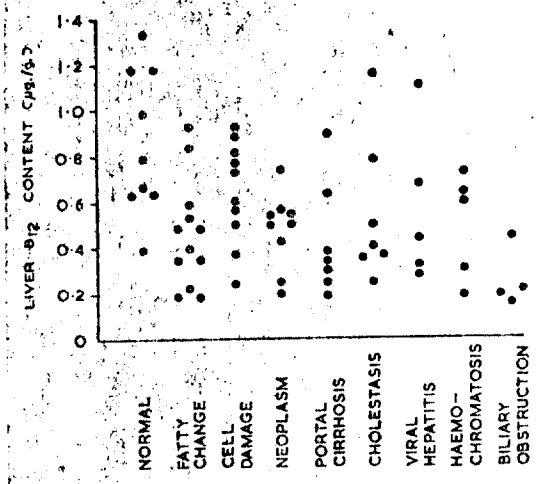

FIG. 1. *Scattergram showing B12 content of liver biopsy specimens in relation to histological diagnosis.*

fatty change. The mean B12 content of these was 0·63 µg. per g. This group included two biopsies from a patient with megaloblastic anaemia complicating anticonvulsant therapy with B12 contents of 0·25 and 0·38 µg. per g. However, this syndrome is probably associated with nutritional deficiency in the majority of cases (Stokes and Fortune, 1958). The remaining eight specimens had a mean B12 content of 0·73 µg. per g., with a range from 0·50 to 0·93 µg. per g., which does not differ greatly from the normal values.

The nine biopsies grouped as metastatic neoplasm include six in which this was demonstrated in the biopsy specimen and three in which it was shown subsequently. The primary site was in the pancreas or gut in seven cases and in the lung in one, the final patient having disseminated reticulosarcoma. Low B12 contents were frequent, the mean of the group being 0·47 µg. per g. It is of interest that all three histologically normal specimens had a low B12 content.

The B12 content was below normal in five of seven patients with portal cirrhosis, the mean of the group being 0·43 µg. per g. Six of the seven were alcoholics while the remaining patient suffered from diabetes mellitus and had a liver B12 content of 0·89 µg. per g. If this patient is excluded, the mean of the other six was 0·35 µg. per g. By contrast, the results in haemochromatosis were slightly higher, the mean of five patients, two of whom were overt diabetics, being 0·49 µg. per g.

The low mean B12 content of biopsies from patients with cholestatic jaundice was largely due to four patients with chlorpromazine jaundice. The highest B12 content from these patients was 0·41 µg. per g. and the mean was 0·35 µg. per g. Cholestasis from other causes had little effect upon liver B12 content.

The low results in patients with viral hepatitis and external biliary obstruction are discussed further below.

Although the relation between clinical diagnosis and liver B12 content has largely been discussed in the preceding paragraphs, in three instances a single clinical condition was associated with a variable histological picture. These were chronic alcoholism, diabetes mellitus, and folic acid deficiency. Liver B12 content was below 0·60 µg. per g. in 13 of 20 biopsies from chronic alcoholics, the mean of the whole group being 0·45 µg. per g. The low results were found in specimens which histologically showed fatty change or portal cirrhosis. On the other hand, six of seven patients with diabetes mellitus had liver B12 contents within the normal range, the mean value being 0·77 µg. per g. Only one of these biopsies was histologically normal. The low result, 0·31 µg. per g., occurred in a severe alcoholic with haemochromatosis.

Four biopsies were obtained from three patients with megaloblastic anaemia due to folic acid deficiency. The B12 content was low in all, the highest value being 0·39 µg. per g. and the mean 0·30 µg. per g.

There was little relation between liver B12 content and serum biochemical values.

The serum bilirubin at the time of biopsy was greater than 1·0 mg. per 100 ml. in 38 of the 70 cases. Although patients with biliary obstruction, cholestasis, or viral hepatitis had high bilirubin levels and low liver B12 contents, there was in general no relation between the two.

The serum glutamic-oxaloacetic transaminase was measured in 49 instances, and the serum albumin in 65 instances. Neither was significantly related to liver B12 content.

The serum alkaline phosphatase was measured in 67 of the 70 cases. In both cholestasis and external biliary obstruction, the serum alkaline phosphatase was raised and the liver B12 content lowered. Thus, 11 of 13 cases with phosphatase levels above 30 King-Armstrong units had liver B12 contents below the normal range, and all of 13 with liver B12 contents greater than 80 µg. per g. had serum alkaline phosphatase levels below 20 King-Armstrong units.

The serum B12 levels were estimated at the time of biopsy in 60 of the 70 cases, and corresponding serum and liver B12 levels in these are shown in Table II. No patient had a serum B12 content below the normal lower limit of 160 µµg. per ml. Slight elevation of serum B12 (>1,000 µµg. per ml.)

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TABLE II

SERUM AND LIVER B12 CONTENTS IN RELATION TO HISTOLOGICAL DIAGNOSIS[1]

Diagnostic Group	*No. of Cases*	*Serum B12 (μμg./ml.)*		*Liver B12 (μg./g.)*	
		Mean	*Range*	*Mean*	*Range*
Normal histology	7	430	160-1,060	0·91	0·39-1·33
Fatty change	12	475	220-770	0·47	0·19-0·93
Parenchymal cell damage	9	630	260-1,840	0·65	0·25-0·93
Metastatic neoplasm	7	5,980	450-20,400	0·51	0·25-0·75
Portal cirrhosis	7	900	330-1,990	0·43	0·19-0·89
Cholestatic jaundice	7	600	290-980	0·55	0·26-1·17
Viral hepatitis	3	1,240	700-1,920	0·57	0·29-1·11
Haemochromatosis	5	870	370-1,410	0·49	0·18-0·73
External biliary obstruction	3	1,100	510-1,940	0·27	0·16-0·45

[1]The results differ slightly from those in Table I because the serum B12 was not estimated in all cases.

occurred in 10 cases, two each of portal cirrhosis, viral hepatitis, and haemochromatosis, and one each of external biliary obstruction, parenchymal cell damage, metastatic carcinoma, and with normal histology. Extreme elevation was found in two patients with hepatic metastases from carcinoma of the pancreas with serum B12 levels of 20,400 and 17,900 μμg. per ml. respectively.

No correlation was apparent between serum B12 levels and liver B12 contents. This may be due to the fact that, although B12 deficiency results in low levels in both serum and liver, liver damage may deplete liver B12 stores while increasing serum B12 levels. An attempt was made to compare the various diagnostic groups with each other by calculating for each patient the ratio of serum to liver B12. With the present units this ratio normally lies below 1,500. It is increased if the serum B12 is increased or the liver B12 is reduced, or both. In the patients with normal liver histology this ratio was within the normal range in all except one in whom it was 15,800 due to a high serum B12 level. The ratio was above normal in all patients with viral hepatitis or external biliary obstruction, but in other groups was extremely variable. The results do not suggest that this ratio would be of value in the differential diagnosis of liver disease.

DISCUSSION

The major theoretical difficulties in the use of liver biopsy specimens for bio-assay are the sampling error inherent in using small amounts of tissue, and the reference standard to which the results are referred.

The first of these is the more serious, and it is probable that in patients where liver disease is not diffuse, such as cirrhosis or metastatic neoplasm, occasional anomalous results will be due to this inherent sampling error. The scattering of results in the present series (Fig. 1) may be in part a reflection of this difficulty. There is, however, evidence to suggest that the variation may be smaller than would first appear. Pitney and Onesti (1961) found agreement between B12 contents of 'biopsy' specimens and tissue blocks in cirrhotic as well as in normal livers. In addition, results within each of the published series are consistent, and, as will be discussed below, the various series agree well with each other.

The problem of a reference standard to which B12 contents may be referred is related to this problem of possible sampling error. Previous workers without exception have used the wet weight of liver as a reference unit, and the present paper follows this convention. This is both for convenience in comparing results of different workers, and because although more sophisticated standards such as the content of D.N.A. have been suggested, they are unfortunately open to similar objections in non-diffuse liver disease as have been discussed above.

Within these limits, the present results may be compared with others reported in the literature. The mean and range of histologically normal specimens are close to those of Swendseid *et al.* (1957) and of Pitney and Onesti (1961) determined on necropsy material. They are somewhat higher than those of Drouet, Wolff, Karlin-Weissman, and Rauber (1951) and Jhala and Gadgil (1960). It is possible that the latter, in India, were dealing with patients whose nutrition was suboptimal. Nelson and Doctor (1958) reported rather higher values from control patients, the mean from 12 subject being 1·94 μg. per g., with a range from 1·41 to 2·58 μg. per g. The difference may be due to different methods of extraction and assay.

In patients with liver disease, the present mean liver B12 content for patients with portal cirrhosis, 0·43 μg. per g., agrees reasonably with the figure of 0·26 μg. per g. reported by Swendseid and her colleagues (1957). In abnormal subjects Nelson and Doctor (1960, 1962) again found liver B12 levels higher than in the present series, but agree in finding low levels in specimens showing cirrhosis, fatty change, cholestasis, obstructive jaundice, and viral hepatitis. Those with fatty change (with or without cirrhosis) and viral hepatitis gave the lowest values.

The combined results of these various investigations allow some general conclusions to be drawn regarding the factors responsible for liver B12 content. Deficiency of vitamin B12 from any cause lowers hepatic B12 stores. This may occur in pernicious anaemia (Drouet *et al.*, 1951), and to a lesser extent in other states of malnutrition, such as alcoholism, since the results in nutritional cirrhosis were lower than those in haemochromatosis. The low liver B12 content in folic acid deficiency is of interest; it may be nutritional in origin, or, more likely, reflect increased tissue demands for vitamin B12 in the presence of subnormal folic acid supply.

Damage to parenchymal cells seems the most probable cause of the low liver B12 levels usual in viral hepatitis, since the serum B12 is often increased in the condition. The lack of correlation between lower liver B12 contents and increased serum glutamic-oxaloacetic transaminase levels does not conflict with this, since glutamic-oxaloacetic transaminase is cleared from plasma rapidly (Dunn, Martins, and Reissmann, 1958) compared with B12. The raised serum B12 level in some patients with cirrhosis probably also reflects continuing parenchymal cell damage. Nelson and Doctor (1960) suggest that, in addition, hepatic capacity to store B12 may be diminished.

The cause of the low liver B12 content in patients with external biliary obstruction is less certain. It is not an artefact due to the presence of bile components in large amounts in the specimen, for higher levels occur in the cholestatic syndrome, especially when due to causes other than chlorpromazine. Parenchymal cell damage and diminished storage capacity seem likely explanations, but further studies are required for a definite answer.

Replacement of parenchymal cells by tissue unable to store vitamin B12 is also important. This applies to fibrous tissue in cirrhosis, to fatty change in the liver (where malnutrition may also be a factor), and to metastatic neoplasm, where the capacity of the neoplastic cells to store B12 is less than that of normal parenchymal cells (Nelson and Doctor, 1962). There are, however, several anomalous findings in this situation. Parenchymal tissue without histological neoplastic infiltration has been found in some instances to have a low B12 content (Nelson and Doctor, 1958; present series). Moreover, there are reports of raised serum B12 levels, often extreme, in patients with various types of neoplasm irrespective of whether the liver is directly involved (Beard, Pitney, and Sanneman, 1954; Grossowicz, Hochman, Aronovitch, Izak, and Rachmilewitz, 1957; Nelson and Doctor, 1962, present series). Other patients with apparently comparable disease show normal or low serum B12 levels. No explanation of these anomalies has been suggested, and metabolism of vitamin B12 in patients with neoplastic disease remains an unsolved problem.

My thanks are due to Dr. W. R. Pitney from whose studies this project was developed, and to my colleagues in the Departments of Haematology and Biochemistry, Royal Perth Hospital, for observations reported in this paper.

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Short Communication

[Agr. Biol. Chem., Vol. 36, No. 1, p. 164~165, 1972]

Microbiological Activities of Coenzyme Forms of Vitamin B_{12}

Sir:

Numerous works have been reported on the microbiological activities of a variety of vitamin B_{12} analogues. For the microbiological determination, vitamins B_{12} are extracted as the cyano forms because of their high extractability and stability. It is today accepted that cobalamin, 5,6-dimethylbenzimidazolyl cobamide, is not only present in but also acts as the coenzyme form, namely, 5′-deoxyadenosylcobalamin (DBCC) or methylcobalamin (CH_3-B_{12}) in biological systems. However, no report has appeared on the microbiological activity of coenzyme forms of cobalamin, although Barker *et al.*[1] reported those of adenyl cobamaide coenzyme for *E. coli* 113-3 and *Ochromonas malhamensis*. On the occasion that methylcobalamin-a (CH_3-B_{12-a}), an isomer of CH_3-B_{12}, was obtained[2] as a by-product of partial synthesis of CH_3-B_{12}, investigation was made on the microbiological activities of CH_3-B_{12}, CH_3-B_{12-a} and DBCC.

Cyanocobalamin (CN-B_{12}) and hydroxocobalamin (OH-B_{12}) (ROUSSEL-UCLAF, Paris), DBCC and CH_3-B_{12} (Eisai Co., Ltd., Tokyo) and CH_3-B_{12-a} prepared by Friedrich's Laboratory were used as test samples. *E. coli* 215 and *O. malhamensis* were used as the test organisms for the microbiological determination of the vitamin B_{12} analogues. The assay procedures employed[3] were those generally used for the both organisms with several modifications. Since coenzyme forms of vitamin B_{12} are sensitive to light and cyanide, every process was carried out without cyanide in the dark. Sample solutions were sterilized by autoclaving at 10 lb for 10 min without or 15 lb for 5 min with the respective basal medium for *O. malhamensis* or *E. coli* 215, respectively, or filtered by passing through Millipore Filter GS followed by mixing with sterilized basal medium.

To convert each coenzyme form of vitamin B_{12} to CN-B_{12}, KCN solution was added in a molar ratio to the vitamin 100:1 to each test solution of coenzyme form of vitamin B_{12} and OH-B_{12} adjusted to pH 6.5, to give the final concentration of the vitamin 10 μg/ml. The solutions of DBCC and CH_3-B_{12} were kept standing in the sun for 2 hr after addition of KCN. The sample solutions thus treated were subjected to microbiological determination after confirmation of conversion to CN-B_{12} by means of UV-visible spectrophotometry.

As shown in Table I, all the B_{12} analogues other than CH_3-B_{12-a} showed almost similar *E. coli* activity to CN-B_{12}, although the activity of OH-B_{12} was comparatively low. It is surprising that the two coenzyme forms, CH_3-B_{12} and DBCC, have a remarkably high *Ochromonas* activity as compared with CN-B_{12}, and on the contrary, OH-B_{12} as well as

TABLE I. MICROBIOLOGICAL ACTIVITIES OF B_{12} ANALOGUES. I

Every process was carried out without KCN.

	Autoclave[a]		Millipore filtration	
	E. coli	*O. malha.*	*E. coli*	*O. malha.*
CN-B_{12}	100	100	100	100
OH-B_{12}	85	28	88	90
CH_3-B_{12-a}	20	27	21	19
CH_3-B_{12}	98	341	95	75
DBCC	90	190	84	56

[a] Autoclaved at 15 lb for 5 min for *E. coli* and at 10 lb for 10 min for *O. malhamensis*.

Each value (mean of more than 10 estimated values) expressed as a percentage of the CN-B_{12} activity.

TABLE II. MICROBIOLOGICAL ACTIVITIES OF B_{12} ANALOGUES. 2

To every B_{12} analogue other than CN-B_{12} was added KCN.

	Autoclave		Millipore filtration	
	E. coli	*O. malha.*	*E. coli*	*O. malha.*
CN-B_{12}	100	100	100	100
OH-B_{12}	97	97	101	114
CH_3-B_{12-a}	93	105	110	104
CH_3-B_{12}	109	111	106	102
DBCC	92	109	104	109

CH_3-B_{12-a} a considerably low activity. On the other hand, millipore-filtration of B_{12} analogues resulted in a much lower activity in cases of CH_3-B_{12} and DBCC, while marked difference was observed in *E. coli* activity between the B_{12} analogues. It is noticeable that CH_3-B_{12-a} did not exhibit an antagonism to CN-B_{12} in *E. coli* and *Ochromonas malhamensis*, but rather showed a microbiological activity to both of the organisms, although it was considerably low. Based on the Friedrich's report,[2] it is supposed that the phenomenon might be partly due to heat conversion of CH_3-B_{12-a} to CH_3-B_{12} and attainment to the equilibrium between the two coenzyme forms.

As shown in Table II, it was confirmed spectrophotometrically as well as microbiologically that addition of KCN converted every B_{12} analogue to CN-B_{12}, regardless of the sterilization condition and the kind of the test organisms.

These findings seem to suggest that autoclave-treatment might have caused some change in the chemical structures of coenzyme forms of B_{12}, probably in the upper ligands, so that the resulting compounds might have more easily passed through the cell membrane of *O. malhamensis*, while the coenzyme forms as themselves are difficult to pass through the membrane, so that they might require more time for incorporation into the cells, or show a lower activity for some other reasons.

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Nutritional vitamin B_{12} deficiency in rhesus monkeys[1,2,3]

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ABSTRACT It has been reported that monkeys deprived of dietary vitamin B_{12} have low serum levels and that some develop "cage-paralysis," i.e., demyelination of the posterior and lateral columns of the spinal cord resembling that in human subacute combined degeneration (SCD). To extend these findings under controlled conditions, 12 monkeys were fed a defined experimental diet that supplied less than 500 pg B_{12} per day (8 were studied for 31 months, 4 for 20 months). Three monkeys received 20 μg B_{12} intramuscularly every 14 days; three monkeys received supplemental folic acid. The deficient animals showed perisistently falling B_{12} levels in serum (to 10 pg/ml by 13 months) and RBC (to 72 pg/ml by 20 months). At 21 months, hepatic B_{12} levels were 5% of controls. After 16 months all deficient animals (but no controls) excreted abnormal amounts of methylmalonic acid. Assays of folate, B_6, iron, ascorbic acid, vitamin E, and trace metals were used to exclude or correct other dietary deficiencies. The monkeys have normal growth rates, appear healthy, and behave normally. Eight to 15 months after marked B_{12} deficiency was documented, peripheral blood and bone marrow show no abnormalities nor is there clinical evidence of SCD. *Am. J. Clin. Nutr.* 27: 470–478, 1974.

The usefulness of animal studies in elucidating the pathophysiology of human vitamin B_{12} deficiency has been limited because of the apparent resistance of experimental animals to the induction by dietary B_{12} deprivation of the full hematological and neurological abnormalities seen in man. Resistance to the induction of B_{12} deficiency may result from coprophagia, preening, ingestion of insects, and difficulty in providing diets with markedly restricted B_{12} content that are otherwise adequate for health. Subhuman species may not readily manifest the overt abnormalities because of lower rates of B_{12} loss from tissues, preferential redistribution of compromised B_{12} stores, diminished dependency upon exogenous supplies, more effective metabolic utilization of available B_{12}, or different metabolic pathways. Additionally, most animal studies have not involved B_{12} deprivation for times comparable to the 4 or more years that are sometimes required for florid deficiency disease to develop in man (1).

Few animals show any features of megaloblastic anemia when subjected to B_{12} deficiency alone, although megaloblastic anemia can be produced readily in several species by folate deficiency alone (2). In some experimental animals (weanling pigs, rats, chicks, guinea pigs, and sheep), vitamin B_{12} deficiency causes a mild to moderate normocytic anemia that lacks the classic morphological alterations of the peripheral blood and bone marrow, the pancytopenia, and the sensitive, orderly reticulocyte response to treatment with B_{12} alone that is characteristic in man (2).

No animal species is known that consistently manifests subacute combined degeneration of the spinal cord[8] when subjected to nutritional

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[8] Subacute combined degeneration (SCD) will be used to refer to the specific myelopathy and other accompanying neurological lesions caused by B_{12} deficiency in man.

deprivation of B_{12}. Captive monkeys sporadically develop demyelination of the spinal cord, a disorder which has been variously described under the label cage paralysis. Some instances of cage paralysis resemble human SCD in both clinical and pathologic features (3–6).

Oxnard et al. (7–12) have attempted to relate cage paralysis to vitamin B_{12} deficiency. These authors described low serum B_{12} levels in a significant proportion of a large colony of rhesus monkeys fed a vegetarian diet. Because of the sporadic occurrence of cage paralysis, some monkeys were killed and found to have demyelination of the posterior and lateral columns of the spinal cord. Despite incomplete descriptions of the clinical, hematological, and histopathological abnormalities in these animals, and the absence of well-defined controls, the topography of the spinal cord lesions was sufficiently suggestive of SCD in association with B_{12} deficiency to warrant further study.

The present observations represent an attempt to *1*) produce, by dietary manipulations under controlled conditions, prolonged pure vitamin B_{12} deficiency in rhesus monkeys, *2*) assay induced alterations of vitamins in blood and tissues, *3*) determine effects on known B_{12}-dependent biochemical processes, *4*) evaluate changes in the bone marrow, blood, and nervous system, and *5*) document that encountered changes are halted or reversed by the administration of B_{12} alone. The study has now been in progress for 31 months.

Materials and methods

Twelve young male rhesus monkeys (*Macaca mulatta*) have been kept on a diet supplying a minimal and nearly irreducible amount of vitamin B_{12}. The animals were housed individually in stainless steel cages fitted with open bar floors to minimize contact with excreta; coprophagia has never been observed. The rooms and the animals have been kept free of insects. Extraneous sources of B_{12} were therefore minimal.

Rhesus monkeys received from an animal farm had tuberculin testing twice and repeated stool examinations for ova and parasites. All animals were fed the identical experimental diet. For purposes of this study, four subgroups were defined by weight and by therapy with B_{12} or folic acid.

Group I: Eight monkeys were received at a mean weight of 4.4 kg (calculated age, 33 months). Group I-A: Five were fed the diet alone. Group I-B: Three received an intramuscular injection of 20 μg hydroxycobalamin every 14 days in addition to the diet.

Group II: In the 11th month of the study, four more rhesus monkeys were added, weighing approximately 2.4 kg each (calculated age, 18 months). Group II-A: One was fed the diet alone. Group II-F: Three received pharmacological amounts of folic acid in addition to the physiological dietary folate (initially, 5 mg folic acid intramuscularly weekly, from the 5th to the 13th month; subsequently, 5 mg folic acid added to the drinking water 5 days/week).

This study used nine B_{12}-deficient animals (with three of these receiving pharmacological amounts of folic acid) and three control animals supplied with physiological amounts of B_{12} by injection.

The animals were initially fed a pelleted diet identical to that used by Dinning and Day (13) except that casein was replaced by isolated soy protein to supply less B_{12}, and the vitamin mix supplied the vitamin E missing from the original diet. The experimental diet[9] consisted of (in grams/kilogram): soy assay protein 170, corn starch 437, sucrose 226, corn oil 80, salt mix 40, baking powder 15, and a vitamin mix (see Table 1). The diet contained 2.5 ng B_{12}/kg. As the larger monkeys consumed 175 g feed/day and the smaller monkeys 115 g feed/day, the daily dietary intake of B_{12} was less than 500 pg. Because deficiences other than B_{12} occurred, the following changes were made:

1) Ferric phosphate was replaced by ferrous sulfate (10th month).

2) Ascorbic acid was increased to 1.5 g/kg before pelleting (19th month).

3) The vitamin mix was enriched (25th month) (Table 1).

4) Vitamin E was increased (29th month) (Table 1).

Vitamins were increased to give the final percentages

TABLE 1
Monkey diet

Vitamin mix	Initial, g/kg[a]	Modified, g/kg[b]
Ascorbic acid	0.2	1.5
Calcium pantothenate	0.02	0.02
Choline chloride	1.0	1.0
Cod liver oil (850 U A/g) (85 U D/g)	30.0	30.0
Folic acid	0.005	0.005
i-Inositol	1.00	1.00
Menadione	0.0044	0.0044
Nicotinic acid	0.02	0.08
Pyridoxine HCl	0.005	0.02
Riboflavin	0.005	0.01
Thiamin HCl	0.005	0.015
Biotin		0.0006
Vitamin E acetate (dry)	(250 IU/g) 0.180	(500 IU/g) 0.45

[a] Diet employed for first 12 months. [b] Final composition from 25th month.

[9] General Biochemicals, Chagrin Falls, Ohio 44022, Catalog No. TD-72449. Salt Mix: (Hubbell, R. B., L. B. Mendel and A. J. Wakeman. New salt mixture for use in experimental diets. *J. Nutr.* 14: 273, 1937.)

by weight used in standard monkey diets (14, 15) to provide adequate and physiological amounts.

Twelve milliliters of venous blood were taken monthly from the femoral vein. Sodium thiamylal 3% was used for anesthesia for blood drawing on monkeys over 7 kg in weight or for minor procedures.

Complete blood counts were done monthly and included RBC count, hematocrit, hemoglobin, reticulocyte count, white blood count, platelet count, and examination of the blood film. Bone marrow aspirations were performed at intervals and examined by Wright's stain and Perl's Prussian blue stain for iron.

Vitamin B_{12} content of serum and red cells was determined monthly by the *Euglena gracilis* Z-strain assay (16). Vitamin B_{12} determinations on liver biopsy specimens (obtained by closed biopsy with a Menghini needle) utilized the same assay after tissue preparation (17). Folate content of serum and red cells was also determined monthly using the *Lactobacillus casei* assay (18, 19).

Methylmalonic acid (MMA) excretion was measured periodically. The monkeys were given 2 g of L-valine in their water and then 4 hr later placed in a metabolic cage without food for an 18-hr urine collection. Because the water is taken slowly and sometimes incompletely, this dosage approximates the physiological intake of valine. The methylmalonic acid excretion in the urine was measured by the method of Giorgio and Plant (20) as modified by Williams et al. (21). In contrast to Williams et al., we found the most accurate fractions for assay were the second and third 10-ml aliquots that eluted from the Dowex column in HCl.

To test the adequacy of the experimental diet, certain vitamins and trace elements were measured. Serum iron (22), serum and red cell pyridoxal phosphate levels (23) were followed serially. Occasional measurements were made of buffy coat ascorbic acid (24), serum vitamin E, red cell susceptibility to lysis by hydrogen peroxide, and blood trace metals (Zn, Cu, Ca, Mg, Na, K).

Neurological examination was made with the monkey restrained in a metal box that allowed evaluation of muscle tone, deep tendon reflexes, cranial nerve function, and response to pin prick. The monkeys were periodically observed and filmed in spontaneous activity in a large, room-sized cage. Their agility and speed in running, climbing, and jumping gave a more sensitive picture of neurological status. Fore-limb coordination was observed by making the monkey manipulate a stick thrust into the cage and by watching the monkey handle food pellets.

Results

B_{12} Assays

The deficient monkeys (groups I-A, II-A, II-F) showed a progressive fall in serum B_{12} to levels near the limit of accuracy of the assay, whereas control animals (I-B) maintained their serum levels in a normal range (Table 2).

The 12 monkeys started with an initial mean red cell B_{12} of 416 pg/ml. The mean red cell B_{12} of the deficient monkeys has fallen to 25% of this value for groups II-A and II-F and 15% of this value for group I-A (Table 2). The red cell B_{12} of the controls (I-B) is unchanged. The red cell B_{12} varies more than the serum B_{12} on sequential determinations.

Tissue B_{12} stores were examined by paired liver biopsies of control and B_{12}-deficient animals from group I done in the 21st and 28th months on the experimental diet. The liver B_{12} of the deficient animals (I-A) was approximately 5% of the B_{12} concentration found in the controls (I-B) (Table 3).

MMA, 0.167 to 0.786 mg/kg per day, was detected in the urines of all deficient animals in 11 out of 20 assays. The first positives were obtained at 20 months in group I-A and at 16 months in group II. The control animals never had detectable MMA in the urine.

TABLE 2
Blood vitamin B_{12}, pg/ml

Months on diet	I-A; 5 monkeys (Diet alone)		I-B; 3 monkeys (Diet + B_{12})		II-A; 1 monkey (Diet alone)		II-F; 3 monkeys (Diet + folate)	
	Mean S B_{12}[a]	Mean RC B_{12}[b]	Mean S B_{12}	Mean RC B_{12}	Mean S B_{12}	Mean RC B_{12}	Mean S B_{12}	Mean RC B_{12}
1	224	704	162	226	125	190	160	228
7	76	262	230	776	40	74	45	119
13	39	89	313	468	10	78	20	111
19	18	44	220	301	10	89	10	115
25	5.5	76	130	368				
31	11	54	371	401				

[a] S = serum. [b] RC = red cell.

TABLE 3
Liver B_{12} in nanograms/gram wet tissue

Months on diet	Monkey	B_{12}	A as percent of B
21	I-A #1[a]	156.3	4.9%
	I-B #3[b]	3,175.0	
26	I-A #7[a]	73.3	4.8%
	I-B #2[b]	1,570.0	

[a] Group I monkeys on vitamin B_{12}-deficient diet. [b] Group I monkeys on vitamin B_{12} supplementation.

Folate assays

The serum folate values for all animals have remained well up in the normal range established for humans in this laboratory with no presently discernible trends and no differences between the B_{12}-deficient groups and the B_{12}-supplemented controls. Similarly, despite individual variations and variations among individuals, the red cell folate levels show no discernible trends and no consistent differences among the various groups.

Other vitamins and minerals were assayed in the blood. Depressions in serum pyridoxal phosphate and vitamin E of indeterminant significance were noted in a few of the monkeys and corrected by enrichment of the vitamin mix. Analysis of trace metals in the serum and whole blood has shown normal levels of Cu, Mg, Na, K, and Ca, but a moderately low value of Zn (70% of the normal human values).

Adequacy of diet

We have attempted to supply adequate physiological amounts of vitamins (other than B_{12}), trace elements, and major nutrients. The monkeys appear healthy (specifically with a normal general effect, coat of hair, gums, tongue, musculature, and appetite). In addition, histological studies of the liver using stains for iron, fat, glycogen, and connective tissue have shown no abnormalities. The group II monkeys have maintained their expected weight. The group I monkeys appear mildly obese and are above their expected weight, with the matched controls (I-B) somewhat heavier than the deficient animals (I-A) (Fig. 1) (15).

As had been anticipated with the use of ferric phosphate (25), the group I monkeys became iron deficient at the 10th month (microcytic hypochromic anemia, low serum iron levels, lack of iron stores in the bone marrow). The anemia responded completely to Imferon alone within 1 month. Normal iron levels and stores have been maintained since by replacing the salt mix iron with ferrous sulfate (0.27%).

The group I monkeys developed florid scurvy in the 12th month of study. Within a few days, the animals had apparent hind limb weakness (manifest by moving in a sitting posture with the hind limbs dragging and by inability to raise themselves onto their low perches), lethargy, dazed effect, periorbital ecchymoses, and rectal bleeding. In retrospect, mild gum bleeding on trauma had occurred for approximately 2 months prior to this. Buffy coat ascorbic acid was undetectable. Parenteral ascorbic acid (100 mg/week) gave complete clinical recovery within 3 weeks. As we found that 30% of the ascorbic acid in the vitamin mix was lost during pelleting, the ascorbic acid was increased to 1.5 g/kg. Ascorbic acid levels in white cells and platelets were normal 90 days after starting the enriched diet.

Hematological studies

The monkeys have had normal blood counts except during the periods of overt iron-deficiency anemia and scurvy (Table 4) (26). Compared with man, *Macaca mulatta* has a smaller MCV and MCHC, and a leukocytosis with elevated lymphocytes (55 to 70%). Periph-

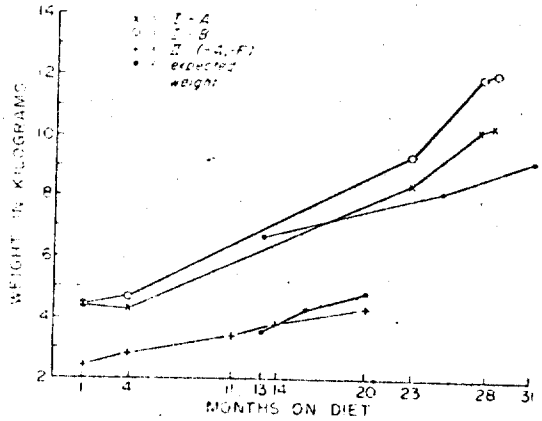

FIG. 1. Growth of monkeys. Group I-A are 5 monkeys on diet only, I-B are 3 monkeys on vitamin B_{12} supplementation, II A-F are 3 monkeys on diet and PGA supplementation. There are no significant differences in the individual groups from expected normal values.

TABLE 4
Complete blood counts on eight group I monkeys

Time on diet	RBC, $\times 10^6/mm^3$	Hb, g/100 ml	Hct, %	MCV, μ^3	MCH, pg	MCHC, g/100 ml	Reticulocytes, %	Platelets, $\times 10^3/mm^3$	WBC, $\times 10^3/mm^3$	Method for RBC and Hct
2nd month										
I-A[a]	5.32	14.5	44.0	84	26.2	30.4	0.76	542	16.2	Hand count
Control I-B[b]	5.91	15.4	48.7	82.6	26.0	31.3	0.77	534	16.8	
11th month										
I-A	6.21	14.2	46.6	75.3	22.9	30.71	0.96	478	15.0	Coulter counter
Control I-B	6.17	14.3	47.0	74	22.9	30.81				
14th month										
I-A	5.41	14.6	45.8	85	27	32.4	0.56	471	10.9	Hand count
Control I-B	5.15	14.6	46	89.2	28.3	32.0	0.50	494	11.4	
31st month										
I-A	5.43	13.5	38.9	74	24.2	33.9	1.16	492	10.1	Coulter counter
Control I-B	5.74	14.9	43.6	75.3	25.8	34.1	1.57	487	10.7	
Normal, 41 *M. mulatta* (26)	6.25	12.7	42	67	20	30	0.83	Not available	15.0	

[a] I-A = 5 monkeys on diet only. [b] I-B = 3 monkeys on diet + B_{12}.

eral blood smears did not show macrocytosis. Occasional 7- or 8-lobed polymorphonuclear neutrophils are seen in the smears of control as well as deficient animals. These are within the normal range for rhesus monkeys. Our mean lobe counts are below the normal of 5 to 6 for rhesus monkeys (26).

All bone marrow aspirations over a period of 2 years have shown active erythropoiesis with normal nuclear maturation. The cytoplasm of some intermediate or late normoblasts appears ragged, but there has been no progression of this mild abnormality. Granulocytic precursors appear normal with no giant forms. Bone marrow iron stains show normal iron stores and no abnormal sideroblasts. Megakaryocytes appear normal without giant multisegmented forms. None of the animals shows morphologic signs of megaloblastic hematopoiesis.

Neurological observations

One B_{12}-deficient monkey developed neurological abnormalities starting in the 21st month, which resolved almost completely after starting the vitamin-enriched diet in the 25th month. This monkey developed a lethargic, apathetic behavior, and weakness of all four limbs affecting gait, posture, climbing, and grip. Knee and ankle jerks were diminished. Although 50 mg thiamin intramuscularly was without benefit, gradual complete recovery including return of normal deep tendon reflexes followed treatment with 5 mg pyridoxine hydrochloride intramuscularly and enriched vitamin mix in the diet. A decline in serum pyridoxal phosphate to 20 ng/ml (normals above 30 ng/ml) had coincided with the illness.

One B_{12}-supplemented animal (I-B) has exhibited intermittent self-mutilation starting from the 24th month of study. This behavior is seen occasionally in monkeys caged in isolation for years and has not been related to dietary deficiencies. By moving the monkey to different cages, the frequency of self-mutilation has been greatly diminished.

None of the 12 monkeys exhibits the hind limb clumsiness and depression of the reflexes which would be expected as early signs of SCD. Aside from the instances described above, all animals have normal behavior and motor function.

Discussion

Vitamin B_{12} deficiency in monkeys

A) Manipulation of diet to induce deficiency. Previous workers have produced low serum vitamin B_{12} levels in monkeys by feeding vegetarian diets (7, 9, 27). Wilson and Pitney (27) used an experimental diet based on casein protein supplemented with carbohydrates, fats, salts, and a vitamin mix adjusted to supply 1 μg of B_{12}/day (5 ng B_{12}/g diet). However, B_{12} deficiency only developed in monkeys receiving either no added folic acid or no added ascorbic acid, so that due to concomitant deficiencies affecting small intestinal absorption, the amount of B_{12} absorbed may have been much less than that offered in the diet. May et al. (28) studied folic acid, ascorbic acid, and vitamin B_{12} deprivation in monkeys fed a milk diet providing 1 μg B_{12}/day. If adequate amounts of ascorbic acid and folic acid were supplied, the monkeys developed no evidence of B_{12} deficiency anemia and maintained normal liver B_{12} stores of 1.0/μg per g wet weight. Oxnard et al. (7, 9) produced low serum B_{12} levels in rhesus monkeys by feeding a diet of dried milk, vegetables, and fruit, supplemented by Bemax, ascorbic acid, and halibut liver oil. The B_{12} content of this diet is not known. We chose to use a well-defined experimental diet to avoid possible variations in the B_{12} supplied and to attempt to supply known adequate amounts of other vitamins. Our diet, using isolated soy protein, supplied less than 500 pg B_{12}/day.

A major difficulty encountered when employing an experimental diet may be the induction of a deficiency other than the one desired. It is also best to avoid pharmacological amounts of dietary components. As described above, the initial experimental diet caused certain vitamin and mineral deficiencies. These were corrected by increasing the vitamin content to the percentages contained in standard monkey diets and by supplying a more absorbable form of iron. Physical examination and laboratory tests support the adequacy of the final experimental diet.

B) Documentation of B_{12} deficiency. The experimental diet has been most effective in causing serum and tissue depletion of B_{12}. The initial mean serum B_{12} of 184 pg/ml of our monkeys was similar to that previously found in 43 recently captured rhesus monkeys (7), but was higher than the mean of 120 pg/ml for the established monkey colony used by Wilson and Pitney (27). The rate of fall of serum B_{12} levels of monkeys on our diet was greater than that reported in Oxnard's study and equal to that seen in the study of Wilson and Pitney.

Tissue levels of B_{12} were measured because a persistently low serum B_{12} may not always define a functionally significant degree of B_{12} depletion. In a random survey of 630 subjects, 8 were found who had repeated serum B_{12} levels between 29 and 100 pg/ml for 10 years without evidence of deficiency disease (29).

Table 5 presents mean blood and liver values of B_{12} from some large published studies for normal rhesus monkeys, normal man, and patients with anemia due to B_{12} deficiency. The B_{12} values for serum, red cells, and liver in our deficient monkeys (I-A, II-A, II-F) have for 8 to 15 months been as low as those of the anemic patients. For more than 6 months, the mean serum B_{12} level of these monkeys has been extremely low (10 pg/ml). In contrast, the B_{12}-supplemented monkeys (I-B) have maintained values comparable to normal rhesus monkeys and man.

MMA excretion is a useful indicator of biochemical dysfunction due to B_{12} deficiency (33). In the absence of the deoxyadenosylcobalamin coenzyme, methylmalonyl-CoA cannot be converted to succinyl-CoA but instead is hydrolyzed and excreted as the free acid. In B_{12}-deficient rats, a qualitative correlation can be made between the depression of liver methylmalonyl-CoA mutase activity and the amount of methylmalonic acid in the urine

TABLE 5
Vitamin B_{12} values from published studies[a]

	Normal rhesus monkey	Normal man	Patients with B_{12} deficiency anemia
Serum B_{12}, pg/ml	223	x = 450 (160–900)	x = 37, 38 (0–130)
Red cell B_{12}, pg/ml	-	x = 213 (110–500)	x = 63 (25–110)
Liver B_{12}, μg/g wet tissue	1.0	1.0	0–0.09 in 21 of 44 0.1–0.19 in 18 of 44

[a] References 7, 28, 30–32.

(34). It is not surprising that the deficient monkeys (I-A, II-A, II-F) sometimes gave negative methylmalonic acid urinary determinations, as only a physiological dose of valine was ingested, whereas other studies have used pharmacological loading doses of valine. Other authors describe considerable variation in repeated determinations of urinary methylmalonic acid from a single animal (21, 34, 35). There was a clear difference between our B_{12}-deficient animals and the controls (I-B); only the deficient animals excreted detectable MMA. Furthermore, all of the deficient monkeys (I-A, II-A, II-F) excreted quantities of MMA per kilogram of body weight in excess of that which is normal for a 70-kg man (up to 0.057 to 0.120 mg/kg per day (21, 33, 36). Thus, a biochemical lesion due to B_{12} deficiency has been demonstrated in the B_{12}-deprived monkeys.

C) Folate and ascorbic acid measurements. Adequate serum folate levels have been maintained by all 12 monkeys. It is interesting that all 12 show serum values well up in the normal range with red cell concentrations somewhat lower than those found in normal humans.

Other workers have observed declining serum B_{12} and liver B_{12} content in scorbutic animals with rapid recovery of B_{12} levels upon treatment with ascorbic acid (27, 28). Our 12 monkeys received essentially no dietary B_{12}. There was no change in serum or red cell B_{12} with the onset of scurvy or with recovery. This supports the hypothesis previously suggested (27, 28) that scurvy affects B_{12} metabolism by interfering in a readily reversible manner with absorption of B_{12}.

D) Hematologic observations. Oxnard and Smith (7–9) and Wilson (27) did not report hematologic data on B_{12}-deficient monkeys, but Oxnard (11) states that no anemia nor macrocytosis was found. After treatment of some monkeys with B_{12}, a slight drop in MCV is said to have occurred (11). May et al. (28) found no effect of B_{12} in preventing or correcting the megaloblastic anemia of folate deficiency or of combined folate deficiency and scurvy in monkeys. Our B_{12}-deficient monkeys presently show no morphological manifestations of megaloblastic anemia in the blood or bone marrow. This confirms the adequacy of dietary folate (2, 28). Several explanations for normal hematopoiesis in the face of B_{12} deficiency are possible: *1*) Deficiency has not continued long enough to cause depletion of B_{12} within the marrow proliferative pool. In humans, 4 years or more of B_{12} deprivation may be required before megaloblastic changes occur (1). *2*) Monkey hematopoietic cells have higher B_{12} stores or replenish stores from the blood at the expense of other tissues. *3*) Monkey B_{12} or folate metabolism, or both, differs from the human so that hematopoiesis is not as completely dependent on vitamin B_{12}. These hypotheses have not yet been tested.

Neurological disease in vitamin B_{12}-deficient monkeys

Vitamin B_{12} deficiency in man can cause subacute combined degeneration of the spinal cord and brain, a syndrome with a characteristic clinical course and specific neuropathology (37, 38). It has been widely acknowledged that there is no acceptable animal model of combined system degeneration. However, Oxnard and associates (8, 11, 12) have recently published a series of papers describing the development of similar neurological lesions in monkeys maintained on a B_{12}-deficient diet.

In their colony of B_{12}-deficient monkeys, only a few developed overt neurological manifestations. These animals first showed clumsiness of gait, especially in the use of the hind feet for gripping or placing. Deep tendon reflexes and plantar responses, present in recently captured animals, were absent. The most severely affected monkey had flaccid paralysis of the hind limbs and tail. The earliest onset of cage paralysis was at 14 months of captivity (8, 11).

Neuropathology was performed on five afflicted animals (serum B_{12} levels 20 to 80 pg/ml) after 1.5 to 26.5 years on the vegetarian diet (8, 11). A severe symmetrical spongiform degeneration of the white matter of the spinal cord was found affecting the posterior columns more severely than the lateral or anterior columns. The process was patchy in vertical extent and maximal at the mid-thoracic level. Patchy severe demyelination occurred in the peripheral nerves, more severe in the lower nerves of the hind limbs and tail. Similar demyelination was found in the central white matter of the cerebral cortex in one animal.

Monkeys that had low serum B_{12} levels ($<$ 20 to 80 pg/ml) but were "normal" to clinical

observation were also studied pathologically. Four out of seven "long-stay" animals, in the colony for 3.5 to 16.5 years, had similar but milder cord and peripheral nerve lesions. Two out of three "short-stay" animals, in the colony 8 months to 2 years, with serum B_{12} levels from 60 to 80 pg/ml also had mild posterior column and peripheral nerve demyelination. All ten clinically normal animals had absent deep tendon jerks, although these are present in recently captive animals. Four animals with cage paralysis were reported to show "considerable objective improvement" with treatment by parenteral cyanocobalamin.

These data of Oxnard et al. (7–12) demonstrate that rhesus monkeys fed a B_{12}-deficient diet reproducibly develop low serum B_{12} levels, sometimes associated with neurological lesions resembling SCD. These observations are incomplete in that: *1)* clinical observations were sketchy, *2)* documentation of B_{12} deficiency did not include serial serum levels, tissue levels, and functional biochemical tests, *3)* the diet was not precisely controlled, *4)* other deficiency states were not excluded, *5)* the time required to produce neurological disease was not well-defined, *6)* the frequency of neurological lesions was not well-defined, and *7)* specific documentation that neurological lesions were halted or improved by treatment with B_{12} alone was lacking.

It is perhaps surprising that none of our 12 deficient monkeys exhibits signs of SCD. Their serum B_{12} levels have been lower than those of monkeys described as having lesions of SCD (8) and the animals have been B_{12} deficient for longer than the three short-stay monkeys with absent hind limb reflexes (8). Probably a longer period of B_{12} deficiency is required to produce subacute combined degeneration in rhesus monkeys. Other possibilities are that: *1)* neurological disease occurs only in a small percentage of B_{12}-deficient animals that are unusually susceptible, *2)* a combined deficiency state is required to cause neurological disease, *3)* vitamin B_{12} deficiency and an unknown factor, present in the natural diet but absent in the experimental diet, are required to cause SCD, or *4)* cage paralysis is unrelated to B_{12} deficiency.

With the availability of these monkeys, documented to be B_{12} deficient by the criteria detailed in this paper but now lacking overt neurological and hematological changes, it will be possible to examine the correlations, or lack of correlations, between clinical and histological expressions and various biochemical, enzymatic, and functional abnormalities that have been described or proposed for vitamin B_{12} abnormalities (unpublished observations).

Conclusions

1) Rhesus monkeys, deprived of vitamin B_{12} by feeding an experimental diet, can be made markedly deficient as judged by blood and liver B_{12} content.

2) A biochemical lesion of B_{12} deficiency was demonstrated by abnormal methylmalonic acid excretion in deficient animals but not in B_{12}-supplemented controls.

3) Despite severely low blood and liver B_{12} levels for 12 to 18 months, no other manifestations of deficiency disease have appeared. There are no megaloblastic changes and no neurological signs of subacute combined degeneration.

4) A well-characterized experimental diet suitable for the induction of vitamin B_{12} deficiency in the rhesus monkey has been described. Inadequacies of the initial experimental diet are specified.

5) When vitamin B_{12} balance is independent of diet, the occurrence and treatment of scurvy has no effect on vitamin B_{12} levels in the blood.

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Vitamin B_{12} Content of Circulating Leukocytes as an Aid in the Differentiation of the Acute Leukemias

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ABSTRACT

From 25 patients with acute leukemia 116 specimens of leukocytes were assayed microbiologically for total vitamin B_{12} to determine if variation in vitamin B_{12} content would help in differentiating the acute leukemias. The mean cell vitamin B_{12} levels ($\mu\mu$g./10^8 cells) in the different types of leukemia were: lymphoblastic 464, myeloblastic 1058 and monocytic 200. Cell vitamin B_{12} levels above the normal range (100-800 $\mu\mu$g./10^8 cells) are suggestive of myeloblastic leukemia. The only elevated cell vitamin B_{12} levels comparable to those found in myeloblastic leukemia were in reticulum cell leukemia, and this type of leukemia was not difficult to diagnose morphologically. Blast cells contained more vitamin B_{12} than mature cells of the same series; there was a significant positive correlation between the percentage of blast cells and cell levels of total vitamin B_{12} in both lymphoblastic and myeloblastic leukemia.

SOMMAIRE

L'auteur a étudié 25 patients présentant une leucémie aiguë. A l'aide de 116 échantillons de leucocytes, il dosa, par méthode microbiologique, le contenu total en vitamine B_{12}, afin d'établir, si des variations en B_{12} aideraient au diagnostic différentiel des divers types de leucémies aiguës. Les taux cellulaires en B_{12} ($\mu\mu$g./10^8 cellules) pour ces leucémies furent en moyenne les suivants: lymphoblastique = 464, myéloblastique = 1058 et monocytaire = 200. Les niveaux cellulaires en vitamine B_{12} dépassant la normale (100-800 $\mu\mu$g./10^8 cellules) semblent accompagner la leucémie myéloblastique.

Des concentrations cellulaires en vitamine B_{12} aussi élevées que dans la leucémie myéloblastique n'existaient que dans la leucémie à cellule réticulaire, et ce dernier type de leucémie ne devrait causer que peu de problème quant au diagnostic morphologique. Les cellules "blastiques" contiennent plus de vitamine B_{12} que les cellules matures de la même lignée: de plus, il existe un rapport très net entre le pourcentage de blastes et les concentrations cellulaires en vitamine B_{12}, aussi bien pour les cas de leucémie lymphoblastique que myéloblastique.

THE acute leukemias are often difficult to differentiate morphologically. Various laboratory tests have been used to assist in specific diagnosis. An investigation of the value of the vitamin B_{12} content of circulating leukocytes in diagnosis of the acute leukemias is described in the following report.

MATERIAL

Twenty-five patients with different forms of acute leukemia were studied. Nine were originally diagnosed as having undifferentiated blast cell leukemia. The morphology of the leukocytes from these cases was studied independently by four hematologists to determine the type of leukemia. They were in agreement in eight of the nine cases. The ninth case was excluded from this investigation, so the type of acute leukemia was known in all 25 cases studied. There were 41 specimens of leukocytes from six patients with lymphoblastic leukemia, 40 specimens from 10 patients with myeloblastic leukemia, and 13 specimens from eight patients with monocytic leukemia. Specimens were collected at intervals during the course of the dis-

TABLE I.—MEAN VALUES OF TOTAL VITAMIN B_{12} IN CIRCULATING LEUKOCYTES IN DIFFERENT TYPES OF ACUTE LEUKEMIA

NORMALS
Range: 100 - 800 $\mu\mu$g./10^8 cells
Mean: 384 $\mu\mu$g./10^8 cells

Type of leukemia	*Number of specimens*	*Percentage of blast or abnormal cells*	*Mean total cell B_{12} ($\mu\mu$g./10^8 cells)*	
Lymphoblastic	9	10 — 25%	316	
	32	>25%	505	
	41			464
	(21	in remission	366)	
Myeloblastic	8	10 — 25%	373	
	32	>25%	1229	
	40			1058
	(19	in remission	282)	
Monocytic	1	10 — 25%	195	
	12	>25%	200	
	13			200
Reticulum cell	22	>50%		1183

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case. Twenty-two specimens from a single case of reticulum cell leukemia will be reported for comparison; this patient has pursued an indolent course without therapy since 1956, and can hardly be considered a case of acute leukemia.

All of the specimens contained more than 10% blast cells. Additional specimens were collected from some of the patients when they were in remission; these samples contained less than 10% blast cells. Vitamin B_{12} values for these additional specimens will be reported for comparison. There were 21 specimens from three of the six lymphoblastic cases, and 19 specimens from three of the 10 myeloblastic cases.

METHOD

The circulating leukocytes were assayed microbiologically for total vitamin B_{12}, using the Z strain of *Euglena gracilis* as the test organism; details of the method have already been published.[1] Total vitamin B_{12} in circulating leukocytes from normal individuals ranges from 100-800 picograms (micromicrograms) per 10^8 cells; the mean for 35 specimens was 384 picograms.

The acute leukemia specimens were subdivided on the basis of the percentage of blast cells present. The percentage of abnormal cells is more pertinent than absolute values, since different dilutions of cell eluate used for assay represented approximately the same number of cells for each specimen. The values given are the mean values of two assays of each specimen.

RESULTS

The mean values of total cell vitamin B_{12} in the different categories are shown in Table I.

Certain observations can be made from Table I. The mean values for the lymphoblastic specimens with less than 25% blast cells, and when in remission, are not substantially different from the mean for normal cells. With more than 25% blast cells the mean value is increased, but is still within the normal range. The mean values for the myeloblastic specimens with less than 25% blast cells, and when in remission, are also not substantially different from the mean for normal cells, but there is a sharp increase in cell B_{12} when more than 25% blast cells are present. This is reflected in the marked difference between the mean value of all lymphoblastic specimens and the mean of all myeloblastic specimens. In monocytic leukemia the values are in the low normal range, and there is no increase in cell B_{12} with increased numbers of monocytoid cells.

Statistical analysis of these figures is complicated by a number of variables: the number of patients in the groups, the number of specimens per patient, the total leukocyte counts, the percentage of blast cells, type of therapy, serum B_{12} levels, etc. The only test considered to be valid was a test of correlation between the percentage of blast cells and the cell B_{12} levels; this test would be valid only if each cell specimen could be considered an independent sample of this relationship. The Spearman rank correlation was indeed highly significant in both lymphoblastic ($r_s = +0.533$, $P < .01$) and myeloblastic leukemia ($r_s = +0.487$, $P < .01$).

Lacking a statistical summary of the data, the detailed results are presented graphically in Fig. 1. This figure shows the relationship between the total B_{12} values of the individual cell samples and the percentage of blast cells in the specimens.

In the *lymphoblastic* group two specimens had B_{12} levels slightly above the normal range. The

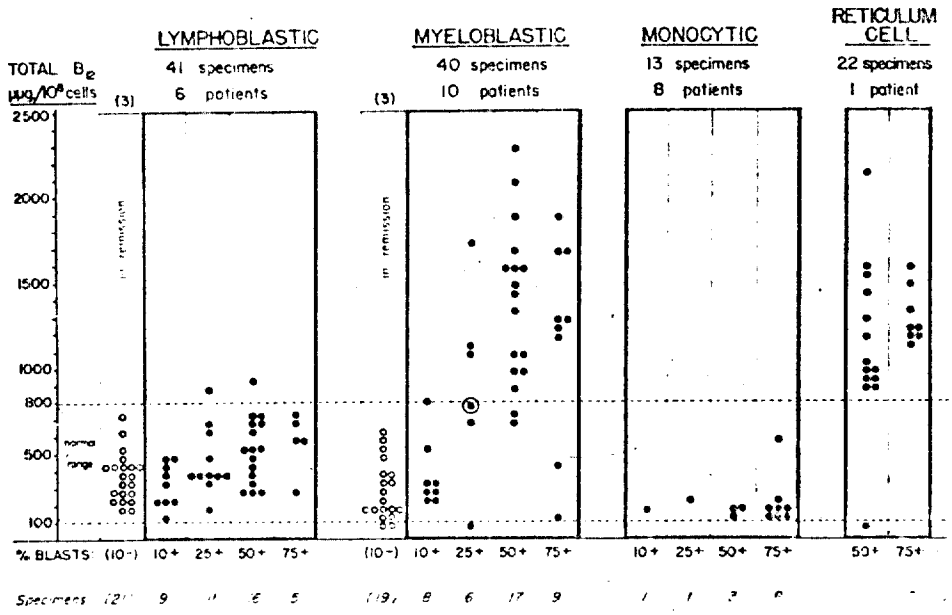

Fig. 1.—Total B_{12} in circulating leukocytes in different types of acute leukemia: distribution of individual values in relation to percentage of blast cells.

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values were normal in all other specimens, irrespective of the number of blast cells present. The 21 specimens collected from three of the patients when in remission also fell within the normal range.

In *myeloblastic* leukemia, when less than 25% blast cells were present, the B_{12} values were normal, and there was little difference in cell B_{12} levels between these samples and specimens collected when the patients were in remission. Above 25% blast cells most specimens showed a marked increase in cell B_{12}, the highest value being just under 2300 $\mu\mu$g./10^8 cells. Twenty-five of the 32 specimens with more than 25% blast cells had B_{12} levels above normal, six were normal, and one was slightly below normal, at 91 $\mu\mu$g.

Is there any explanation for the normal levels in these seven specimens? The two very low levels and another value of 430 $\mu\mu$g. were obtained on single specimens collected two and three days before death. The effect of the terminal state on cell B_{12} levels is not known. The specimen circled in Fig. 1 had 37% blast cells and a B_{12} level of 765 $\mu\mu$g. This was obtained from a patient who presented as a case of chronic granulocytic leukemia and terminated as acute leukemia. Only this one specimen was obtained when he was in the acute stage. Seventeen specimens collected earlier, when his disease was "chronic" or in remission, had cell B_{12} values ranging from 51-243 $\mu\mu$g./10^8 cells, with a mean of 118 $\mu\mu$g., so this value of 765 represents a considerable increase in cell B_{12} for this particular patient. The remaining three high normal values were isolated readings in patients whose other specimens showed elevated B_{12} levels.

No patients with *monocytic* leukemia were seen in remission. Values for most of the specimens fell in the low normal range.

The only increased cell B_{12} levels comparable to those found in myeloblastic leukemia were in the patient with *reticulum cell* leukemia, and this type of leukemia is not difficult to diagnose morphologically. Twenty-two specimens have been collected over a six-year period; all contained more than 50% reticulum cells, and with the exception of one low value for which we have no explanation, cell B_{12} levels have always been greatly increased.

CONCLUSIONS

Two conclusions can be drawn from this study: (1) Blast cells contain more vitamin B_{12} than mature cells of the same series; there is a significant positive correlation between the percentage of blast cells and cell levels of total B_{12} in both lymphoblastic and myeloblastic leukemia. (2) If an acute leukemia specimen contains more than 25% blast cells, and the cell level of total B_{12} is more than 800 $\mu\mu$g./10^8 cells, it is highly probable that it is a case of myeloblastic leukemia.

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The Level of Vitamin B_{12} in Circulating Leukaemic Leucocytes

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ALTHOUGH there is considerable information on serum levels of vitamin B_{12} in leukaemia and allied diseases, there are few data on the levels of B_{12} in leukaemic cells. An investigation of cell levels offered some hope of finding an explanation of the greatly elevated serum B_{12} in chronic granulocytic leukaemia, as it had been postulated that the increased serum levels might be due to the release of B_{12} from circulating leukaemic cells. In addition, morphological and other observations in leukaemia strongly suggest an abnormality of nucleoprotein metabolism; if serial studies of cell B_{12} during the course of the disease showed any significant alterations in response to therapy or with a changing clinical status it was felt that the metabolic disorder might be elucidated. Finally, it was hoped that differences in cell B_{12} might help in distinguishing between the different types of acute leukaemia and be of assistance in planning therapy.

The vitamin B_{12} content of the cells was determined by biological assay. Concomitant serum B_{12} assays were done; these agreed with values already established and will not be reported unless pertinent to some particular case. Diagnosis was by routine haematological methods including marrow aspiration in all abnormal subjects. Emphasis was placed on studying cell levels during the course of the disease, rather than assaying a number of isolated specimens.

METHOD

A modification of the serum assay method of Mollin and Ross (1952) and Ross (1952) was used. The 'Z' strain of *Euglena gracilis* was the test organism (Hutner, Bach and Ross, 1956). The handling of the stock cultures, setting up of the assay, inoculation and incubation of the unknowns conformed to the techniques of the above-mentioned authors. Standard curves were constructed for each assay batch using eight concentrations of a B_{12} solution of known potency. The concentrations used ranged from 0.25 to 10.0 μμg. of B_{12} per ml. Results were read at 650 Å on a Fisher electrophotometer. A rectangular micro-cell of 4 ml. capacity and 10 mm. path length was used. The optical density readings so obtained were converted to μμg. of B_{12} per ml. from the standard curve, and the necessary calculations made to express the results as μμg. of B_{12} per 10^8 cells. All equipment used which was in direct or indirect contact with the specimens was subjected to special B_{12} decontamination cleaning. The main adaptation of the basic method of Mollin and Ross concerned the preparation of cells for assay.

Glassware

All new glassware, and used glass after preliminary cleaning, was boiled in soap and water for 20 minutes, then autoclaved twice in distilled water at 10 lb. pressure for 10 minutes, dried, prepared for use, and finally sterilized by standard methods.

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Collection and Preparation of Sera and Cells for Assay

1. All specimens were collected in specially cleaned glassware. Approximately 30 ml. of venous blood were taken for B_{12} assays, plus an additional aliquot for routine haematological studies.

2. Nine millilitres of venous blood were placed in each of two tubes containing 1 ml. of 5 per cent sodium sequestrene anticoagulant. The balance of the specimen was put in a dry sterile tube; after clot retraction the serum was removed and stored at −10° C. until assayed.

3. The two sequestrene-treated tubes were placed in a 37° C. incubating bath for 1 hour, or until the red cells had sedimented.

4. Plasma was removed from the two tubes with a Pasteur pipette and pooled.

5. The level of the plasma in the combined tube was marked with a grease pencil.

6. Plasma was centrifuged at 270 *g* (1500 rev. min.) for 4 minutes.

7. Supernatant plasma was discarded.

8. 0.85 per cent physiological saline was added to the pencil mark on the tube, the tube contents were well mixed, then centrifuged at 270 *g* for 4 minutes.

9. The supernatant saline was discarded.

10. 0.3 per cent physiological saline was added to the mark (for lysis of any remaining red cells), the contents were well mixed and the tube was allowed to stand for 15 minutes at room temperature.

11. Tube was centrifuged at 270 *g* for 4 minutes.

12. The supernatant saline was discarded.

13. 0.85 per cent physiological saline was added to the mark, the contents were mixed, and the tube centrifuged at 270 *g* for 4 minutes.

14. The supernatant saline was discarded.

15. 0.85 per cent physiological saline was added to the mark. The specimen was now at the *cell suspension* stage, and a leucocyte count was done in the usual manner.

16. Cell suspensions were stored at 4° C. until the eluates were prepared.

The above steps were carried out immediately the specimen was collected. If the leucocyte count was very low the cell suspension was concentrated by centrifuging, removing some of the supernatant, mixing well and repeating the cell count. Cell counts above 2000 per cu.mm. are usually acceptable, and adjustment of the volume of the cell suspensions was avoided whenever possible.

Preparation of Cell Eluates

The cell eluates were prepared within 7 days of collection, and usually within 3–4 days, as follows:

1. The cell suspension was thoroughly mixed and divided into two aliquots, one for assay of free vitamin and one for assay of total B_{12} in cells. The volume in each tube was marked, the tubes corked, and the corks tied down with brown paper caps.

2. One aliquot was heated to 100° C. for 15 minutes for total B_{12} assay, then centrifuged and the supernatant removed and labelled TOSN (total original supernatant). If the white cell count of the suspension was over 100,000,000 cells per ml. the specimen was diluted with a measured amount of distilled water before heating.

3. Distilled water was added to the sediment to reconstitute the original volume and the preparation again heated to 100° C. for 15 minutes, centrifuged and the supernatant removed.

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During development of the method for processing of cells to the eluate stage control measures were carried out. The various saline washes were assayed and yielded no or negligible amounts of B_{12}. Sediments have been reconstituted to the original volume, counts of intact cells attempted and stained smears of the sediment examined for intact cells. Counts on reconstituted sediments can be misleading; what appear to be intact cells in the counting chamber are only large nuclear fragments when seen on stained films. Even though nuclear fragments are large enough to be confusing in the counting chamber they do not yield any significant additional B_{12} after further homogenization by freezing and thawing.

A few platelets are usually seen when the cell count is being done, but the B_{12} content of platelets is low (Kidd, unpublished data), and the presence of a few platelets may be ignored. If many platelets are present steps 13 and 14 under Preparation of Cells for Assay may be repeated as often as necessary to reduce the platelet content of the suspension.

TABLE I

EFFECT OF STORAGE AT 4° C. OF CELL SUSPENSIONS* ON TOTAL CELL B_{12}

Diagnosis	*Days between collection of cells and preparation of eluates*	*Total B_{12} µµg./10^8 cells*
Chronic lymphocytic leukaemia	11	262
	69	260
Monocytoid leukaemia	0	163
	41	167

* Aliquots of the same sample were processed at different times after collection.

Storage of Cells

Cell suspensions in normal saline were formerly stored at 4° C. and the cell eluates prepared just prior to assay. Some very high results were obtained with this method of handling cells. Cells from which eluates were prepared within a few days of collection did not show these abnormally high values. It is possible that the high values obtained when suspensions were stored too long at 4° C. were due to B_{12}-producing bacteria (Dr. E. Lester Smith, personal communication). Some of the patients with these high value cells had obvious clinical infections, suggesting that bacteriaemia was the cause of contamination of the cell suspensions. A few of the cell suspensions on these same patients were grossly contaminated and were discarded before assay. Finally, it was possible to show that long storage *per se* at 4° C. did not cause an increase in total cell B_{12} (Table I).

Re-extractions of Sediments after Total B_{12} Assay

A second extraction of the total sediment (TSSN-1 eluates) has been done on 43 specimens. Expressed as percentages of the first extraction, the values ranged from 0–30 per cent and averaged 11.1 per cent. Undoubtedly incomplete aspiration of the eluate accounts for some of this 11 per cent. A third extraction was done on 30 of the 43 samples and averaged 3.3 per cent of the original extraction.

All the values reported in the tables and figures of results are based on first extractions only, and represent 85–90 per cent of the total cell B_{12}.

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Free Cell B_{12}

All free B_{12} levels are reported as a percentage of the total B_{12} in the cells of the same specimen.

Heating of cell suspensions to 56° C. for 30 minutes lysed the majority of the cells, but did not appear to release any bound B_{12}. The degree of cell lysis at this temperature has been checked by reconstituting the sediments to their original volumes, heating to 100° C. for 15 minutes, and assaying the supernatant for total B_{12}. Our reasoning is that if all the cells are lysed by heating for 30 minutes at 56° C. the bound B_{12} will be in the eluate but will be unavailable to the test organism. Control assays of free sediments for total B_{12} have been done on 36 specimens and the mean value was 23 per cent of the first extraction of total B_{12}. Considered in relation to the mean value of 11 per cent obtained from the first re-extraction of the total sediments, we believe that approximately 12 per cent of the cells are not lysed by heating to 56° C. for 30 minutes.

TABLE II

MEAN TOTAL CELL B_{12} IN NORMAL SUBJECTS AND IN THE DIFFERENT TYPES OF LEUKAEMIA

Type	*Number of cases*	*Number of specimens*	*Mean total cell B_{12} μμg./10^8 cells*
Normal subjects	8	35	384
Chronic lymphocytic leukaemia	9	56	328
Chronic granulocytic leukaemia	14	121	144
Chronic granulocytic terminating in blast cell leukaemia	1	3	327
Acute (blast cell) leukaemia	6	57	668
Reticulum-cell leukaemia	1	9	1083
Monocytoid leukaemia	3	11	190
Total	42	292	

Effect of Chemotherapeutic Agents on the Test Organism

As many of the samples were collected during periods when patients were on active therapy with Myleran and 6-mercaptopurine, the effect on the Euglena of these two drugs was tested. The drugs were added to standard tubes containing 5.0 μμg. of B_{12} per ml. The Euglena organism was exposed to six concentrations of Myleran ranging from 2.5 to 0.0625 μg. per ml., and to four concentrations of 6-mercaptopurine ranging from 2.5 to 0.25 μg. per ml. There was no significant alteration of B_{12} level in any of the tubes, indicating that the growth of the Euglena was unaffected by these agents at these concentrations.

MATERIAL

A total of 370 cell specimens was collected from 34 patients and eight normal subjects, all adults. Fourteen specimens (4 per cent) were grossly contaminated and discarded before assay, and 64 specimens (17 per cent) had abnormally high values and the results were excluded. The 14 grossly contaminated specimens were obtained from nine patients, all of whom had other specimens with high values which were excluded from the analysis. Altogether, 18 patients had some results which were discarded. No specimens have had to be

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discarded because of gross contamination or abnormally high levels since the method of handling cells was changed in November 1958.

Analysis of the assays of total vitamin B_{12} on the remaining 292 cell samples is presented. Sufficient material for assay of free cell B_{12} was available in 273 of these specimens.

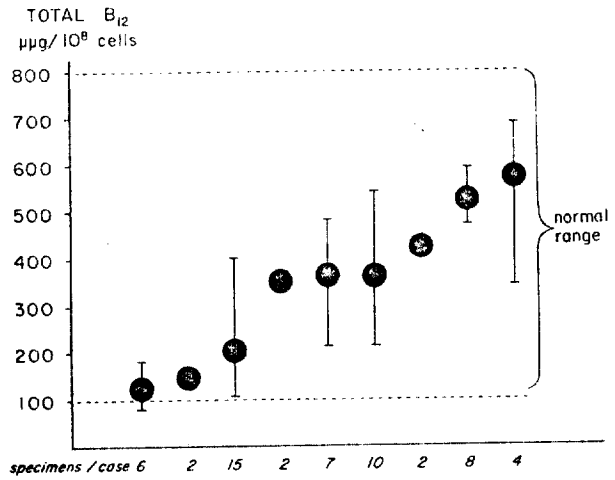

FIG. 1. B_{12} concentration in 56 specimens of white cells from nine cases of chronic lymphocytic leukaemia; the mean value was 328 μμg./10^8 cells. The circles show the mean values and the lines from the circles the range of values in each case.

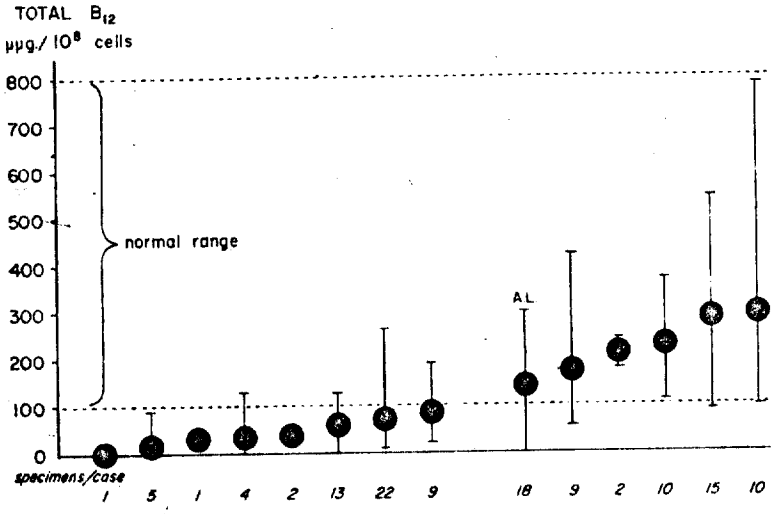

FIG. 2. B_{12} concentration in 121 specimens of white cells from 14 cases of chronic granulocytic leukaemia; the mean value was 144 μμg./10^8 cells. The circles show the mean values and the lines from the circles the range of values in each case.

RESULTS

Total B_{12} in Cells

The mean total cell B_{12} levels in normal subjects and in patients suffering from the various leukaemias are shown in Table II.

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The eight normal subjects had individual values ranging from 102–770 μμg. per 10^8 cells, with a mean value of 384. We have therefore taken 100–800 μμg. per 10^8 cells as the normal range of total vitamin B_{12} in cells as determined by this method.

Fig. 1 illustrates the range of values in nine cases of chronic lymphocytic leukaemia. The mean levels of each case all fell within the normal range, and with the exception of one value of 80, the levels for the individual specimens were within the normal range.

Fig. 2 illustrates the range of values in 14 cases of chronic granulocytic leukaemia. These 14 cases fell into two fairly distinct groups, the eight cases on the left in Fig. 2 having mean levels below 100, with only isolated observations above that level, while the other six cases had mean values within the normal range. Case A. L. had cell values which were below

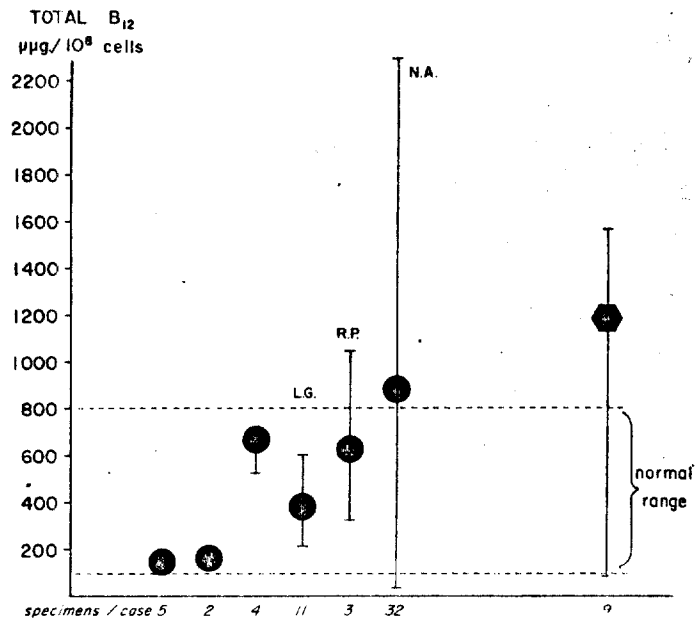

FIG. 3. B_{12} concentration in 57 specimens of white cells from six cases of acute leukaemia and one case of reticulum cell leukaemia. The mean value in the cases of acute leukaemia was 668 μμg./10^8 cells and in the case of reticulum cell leukaemia was 1083 μμg./10^8 cells. The circles and the lines from each circle show the mean values and the range of values for acute leukaemia cases. The hexagon illustrates the case of reticulum cell leukaemia.

normal for 5 months while she was in remission. She then developed haemolytic anaemia, and the spleen was removed. For 10 months after splenectomy until she died, all the cell values were within the normal range. Apart from Case A. L., there were no clinical, haematological or therapeutic differences to distinguish the eight cases with low mean cell levels from those with normal cell values.

The case on the extreme right of Fig. 2 had cell values ranging from 96–784 μμg. per 10^8 cells. This patient had cell values about 300 for the greater part of the 18 months that she was studied. Terminally she had values between 600 and 800 in three samples which contained between 45 and 75 per cent of basophils; earlier differential counts had not shown more than 15 per cent of basophils.

It is apparent from Fig. 2 that values in the upper half of the normal range are rare in

chronic granulocytic leukaemia, and the division of cases into those with low mean levels and those with normal levels may not be justified.

The patient with chronic granulocytic leukaemia whose disease terminated as a blast cell leukaemia (Table II) had a cell value of 43 μμg. per 10^8 cells initially, a value of 113 when in remission, and a final value of 830 when he was in the blast cell phase. The interim value of 113 was found in a sample obtained 6 months before the disease became acute.

Fig. 3 illustrates the range of values in six cases of acute (blast cell) leukaemia, and in one case of reticulum cell leukaemia.

TABLE III

PERIPHERAL BLOOD LEUCOCYTE COUNTS, SERUM B_{12} AND CELL B_{12} VALUES IN THREE CASES OF 'MONOCYTOID' LEUKAEMIA

Case	Date	*Peripheral blood*		*Serum B_{12}*		*Cell B_{12}*	
				μμg./ml.		μμg./10^8 cells	
		Leucocytes (per cu.mm.)	*Abnormal cells (%)*	*Free*	*Total*	*Free*	*Total*
1.	January 7th, 1959	198,000	Blasts—9 early unidentified—74	270	6200	60	165
2.	March 12th, 1959	58,750	Monocytes—55 Promonocytes—25	370	1020	35	240
3.	May 20th, 1958	70,000	67 *	20	310	10	170
	May 23rd, 1958	32,000	– *	10	360	0	315
	June 10th, 1958	17,000	52 *	10	460	20	100
	June 17th, 1958	18,500	– *	30	440	10	260
	July 8th, 1958	7,700	29 *	10	400	40	240
	July 15th, 1958	9,650	25 *	40	410	25	200
	October 27th, 1959	53,000	39 *	20	2500	0	140
	November 6th, 1959	63,750	23 *	20	2400	0	110
	November 10th, 1959	50,000	21 *	20	2600	0	150

*The abnormal cells in Case 3 were difficult to classify, being diagnosed as monocytes or promyelocytes on different occasions. Blast cells never exceeded 4 per cent. Case 3 is perhaps better classified as promyelocytic leukaemia, though the initial normal serum B_{12} is not typical of the granulocytic leukaemias. Cases 1 and 2 were untreated. In Case 3, 6-mercaptopurine was started on May 15th, 1958, and splenic radiation administered in July 1958 and July 1959.

The patient with reticulum cell leukaemia had the highest mean cell value of any case studied. With the exception of one low value for which we have no explanation, all the cell levels for a period of nearly 2 years have been well above the normal range (see also Table V).

In routine haematological diagnosis in our laboratory no attempt is made to subdivide the acute (blast cell) leukaemias, but the slides of these six cases were reviewed independently by four haematologists who were unaware of the cell B_{12} levels. The two patients on the extreme left of Fig. 3 were considered to have features suggestive of monocytoid leukaemia; this correlates with the mean cell level of 190 for the three cases of monocytoid leukaemia shown in Table II. The third patient from the left, with a high normal mean level, was thought to be a case of lymphoblastic leukaemia. These three patients each had a fairly narrow range of values in their individual specimens. All three patients had between 60 and 80 per cent of blast cells in all specimens.

The other three cases of acute leukaemia shown in Fig. 3 were considered to be myeloblastic. The mean level in Case L. G. was normal, but the range of values was wider. The percentage of blast cells in her specimens ranged from 1–23 per cent. The other two cases of

myeloblastic leukaemia, R. P. and N. A., both showed a very wide range of values, the above normal levels coinciding with high proportions of blast cells in the specimens (see also Fig. 5).

The three cases of monocytoid leukaemia listed in Table II were not comparable, and no

TABLE IV

PERCENTAGE OF SPECIMENS WITH MEASURABLE FREE CELL B_{12}, AND THE MEAN FREE CELL B_{12} VALUES IN NORMAL SUBJECTS AND IN LEUKAEMIA

Diagnosis	*Number of specimens*	*Specimens with measurable free B_{12} (% of total)*	*Free B_{12} (% of total cell B_{12})*	
			Mean of positive values	*Mean free B_{12} for all specimens*
Normal subjects	32	6	5.8	0.36
Chronic lymphocytic leukaemia	55	27	18.7	5.1
Chronic granulocytic leukaemia	116	17	6.3	1.1
Acute (blast cell) leukaemia	51	73	33.0	24.0
Reticulum-cell leukaemia	9	89	15.5	14.0
Monocytoid leukaemia	10	70	15.0	10.5
Total	273			

TABLE V

PERIPHERAL BLOOD FINDINGS AND CELL B_{12} LEVELS IN ONE CASE OF RETICULUM-CELL LEUKAEMIA*

Date	*Peripheral Blood*		*Cell B_{12}*		*Free cell B_{12} (percentage of total cell B_{12})*
			μμg./10^8 cells		
	Leucocytes (per cu.mm.)	*Reticulum cells (% of total leucocytes)*	*Free*	*Total*	
March 1956	9,200	30	—	—	—
April 1957	12,100	51	—	—	—
May 1958	15,800	61	—	—	—
October 1958	17,300	68	260	940	28
November 1958	14,000	64	36	970	4
December 1958	18,000	66	50	1150	4
February 1959	22,800	72	60	1000	6
May 1959	16,500	73	70	1580	4
August 1959	18,900	65	340	1520	22
October 1959	14,000	75	0	80†	0
January 1960	15,300	62	—	1280	—
March 1960	17,700	82	230	1230	19
June 1960	15,400	84	550	1490‡	37

* The patient was a man of 40 years.
† No explanation has been found for this isolated low value. Cultures of the cell eluates were sterile.
‡ Not included in mean levels shown in Table II.

conclusions could be drawn. They were separated from the acute (blast cell) leukaemias because of difficulties of classification. In two cases single specimens were obtained from two patients in the final week of their disease after illnesses of 2–3 months. The other nine specimens were from the third patient who was followed for 18 months (Table III).

Free B_{12} in Cells

All free B_{12} cell levels are reported as a percentage of the total B_{12} in the cells of the same specimen so that comparisons can be made between cases or specimens with different total B_{12} values.

The levels of free vitamin B_{12} in cells in normal subjects and in different types of leukaemia are summarized in Table IV.

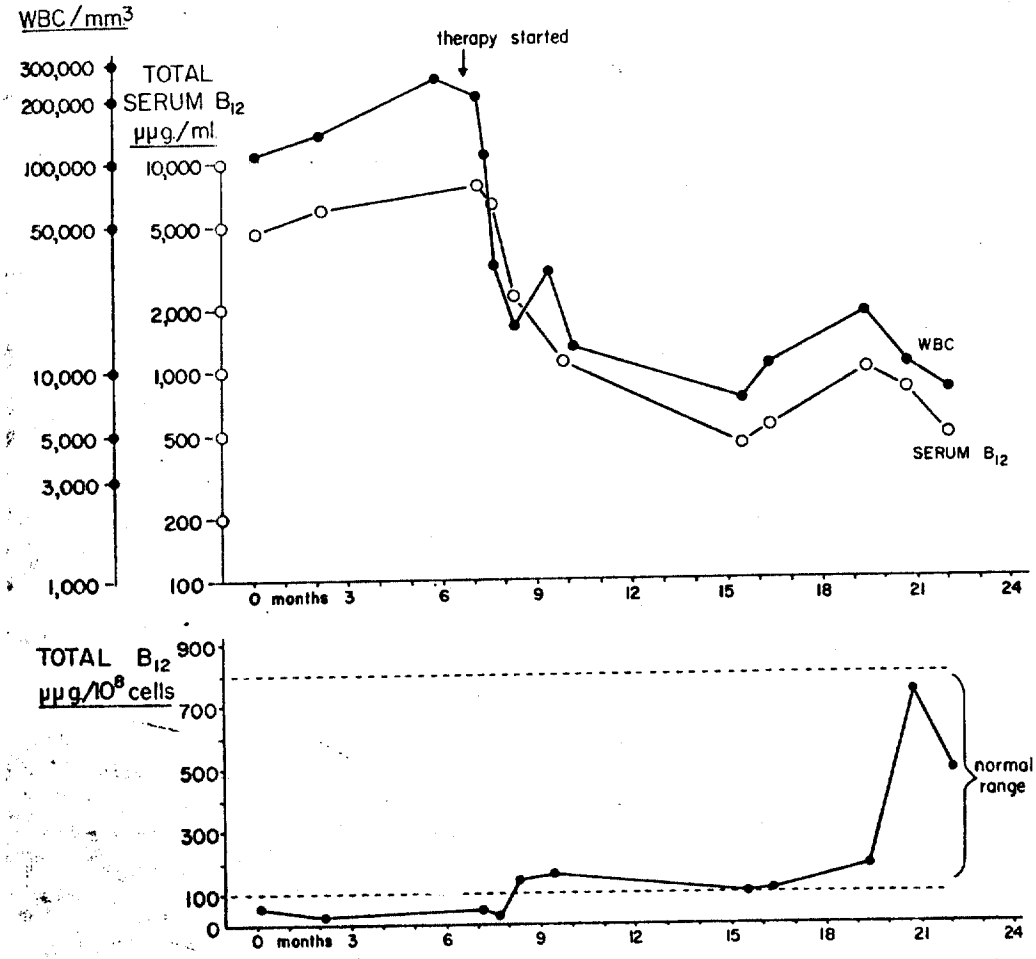

FIG. 4. Observations in a patient with chronic granulocytic leukaemia (male (A. N.) aged 28) showing a good response to therapy. The vertical scale is logarithmic in the upper graph and is linear in the lower graph. (The last two cell-B_{12} assays are not included in the values shown in Table II, or Fig. 2.)

Measurable levels of free cell B_{12} were uncommon in normal subjects and in cases of chronic granulocytic leukaemia. In chronic lymphocytic leukaemia free B_{12} cell values greater than 10 per cent of the total value were more usual when the disease was out of control. More frequent and higher free values were found in the acute leukaemias.

The one case of reticulum cell leukaemia which has been studied was discovered accidentally in March 1956. The patient has been seen at intervals but no therapy has been given.

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B_{12} studies have been done since October 1958. Free and total serum B_{12} levels have been normal at all times (Table V).

DISCUSSION

In general, patients studied over a short period of time, or in a clinically stable state, did not demonstrate wide ranges of cell values, though there was considerable variation in mean cell levels of different patients with the same type of leukaemia.

The most marked changes occurred in the acute and chronic granulocytic leukaemias. Four of the 14 patients with chronic granulocytic leukaemia provided had only one or two specimens. Of the remaining 10 patients, three showed no significant alteration in serum or cell B_{12} levels during the time they were studied even though they had satisfactory haematological responses. In five patients there was considerable reduction, though not to normal levels, in the total serum B_{12}, without any significant alteration in the cell values. They also had satisfactory haematological responses. Case A. L., already mentioned, had an increase in total cell B_{12} to normal levels following splenectomy; she was first studied 6 months after treatment was started and her serum B_{12} levels were normal at all times. Only Case A. N., presented in Fig. 4, showed a complete reversion of serum and cell B_{12} values to normal when in remission.

Total cell B_{12} levels for all specimens of each type of leukaemia were plotted against the peripheral leucocyte counts and against the total serum B_{12} levels. No inter-relationships were observed between the serum B_{12} and the cell B_{12} levels; or between the number of circulating cells and the cell B_{12} values. However, if all the specimens from the chronic granulocytic and acute (blast cell) leukaemia patients were subdivided by the percentage of blast cells in the specimens, there was a striking difference, as shown in Table VI. This point is further illustrated by Case N. A., shown in Fig. 5.

The low mean level of total vitamin B_{12} in the cells of chronic granulocytic leukaemia does not support the hypothesis that the high serum levels of B_{12} are due to the release of the vitamin from the leukaemic cells, but neither does it disprove it.

The finding of increased total cell B_{12} in a case of chronic granulocytic leukaemia when a high proportion of basophils appeared in the specimens prompted the assay of two specimens with marked eosinophilia from cases of Hodgkin's disease. In one specimen with 75 per cent of eosinophils the total cell B_{12} was too low to measure; in the other with an eosinophilia of 40 per cent the total cell B_{12} was 60 μμg. per 10^8 cells, well below normal. These low values may be characteristic of Hodgkin's disease, and not a reflection of the predominant cell type, but it suggests that assays of pure cell cultures would be of interest.

The studies on the six cases of acute leukaemia support the idea that assays of cell B_{12} might assist in the differentiation of blast cell leukaemias, and more acute cases are being investigated to try to establish this.

The high levels of cell B_{12} in the acute (blast cell) leukaemias and the case of reticulum cell leukaemia are of particular interest as the patient with reticulum cell leukaemia is pursuing a benign course.

Various hypotheses could be advanced to explain the high B_{12} levels in leukaemic blast cells. Such levels could be normal for these cells, could be abnormally high due to failure of cell development, or could be due to the disordered function of other substances such as folic acid derivatives.

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SUMMARY

1. A relatively simple method has been described for the biological assay of free and total B_{12} in leucocytes of the peripheral blood.

2. The normal range of total cell B_{12} by this method is 100–800 μμg. per 10^8 cells.

3. Total cell B_{12} levels were found to be normal in chronic lymphocytic leukaemia and mean values were low in chronic granulocytic leukaemia, and high in acute leukaemia and reticulum cell leucaemia.

TABLE VI

PERCENTAGE OF BLAST CELLS RELATED TO THE MEAN TOTAL B_{12} LEVELS IN 21 CASES OF LEUKAEMIA*

Number of specimens	*Blast cells (% of cells in specimen)*	*Mean total cell B_{12} μμg./10^8 cells*
139	Less than 10	154
42	10 or more	837

* Fourteen chronic granulocytic, 1 chronic granulocytic terminating in blast cell leukaemia and 6 acute blast cell leukaemias.

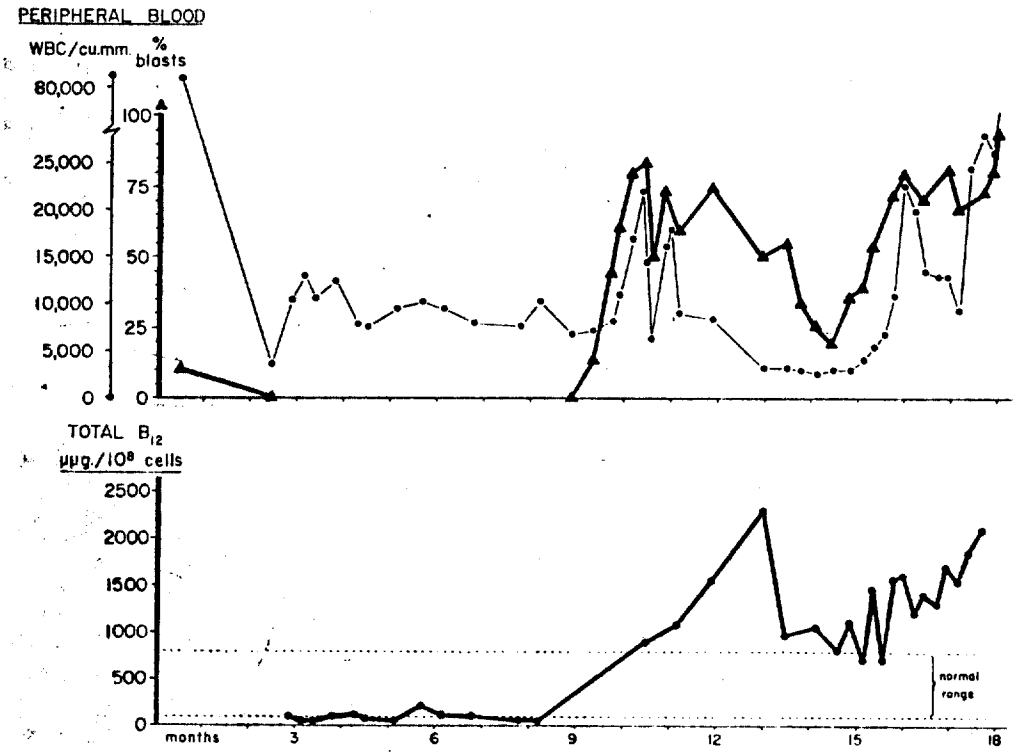

FIG. 5. Observations in a patient (N. A., female, aged 25) with myeloblastic leukaemia. The figure shows the relationship between the total B_{12} in the cells and the percentage of blast cells in the specimens.

Upper part of graph
●——● = white cell count
▲——▲ = percentage of blast cells

4. Total cell B_{12} levels were high when there was a high proportion of blast cells in the specimens.

5. Measurable levels of free cell B_{12} were found sporadically in normal subjects and in patients with the chronic leukaemias, and were usually less than 10 per cent of the total cell B_{12}.

6. Higher free levels were more frequently found in chronic lymphocytic leukaemia when the disease was out of control.

7. Measurable free levels were present in the majority of specimens in the acute leukaemias and in one case of reticulum cell leukaemia.

8. No relationship was found between serum and cell levels of B_{12}, or between the cell levels of B_{12} and peripheral leucocyte counts.

9. The therapeutic response in the various leukaemias could not be predicted from the initial total cell B_{12} level.

ACKNOWLEDGMENTS

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Free and Total Vitamin B_{12} in Cerebrospinal Fluid

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ABSTRACT

Free and total vitamin B_{12} levels in serum and cerebrospinal fluid (CSF) were bioassayed, since there were no available data on the relationship between free and total vitamin B_{12} in CSF or between free vitamin in serum and CSF vitamin B_{12}. The subjects were 43 neurological patients. Serum levels were normal in 40 of 43 patients. Values for free and total vitamin B_{12} in CSF were the same in 42 of 43 patients. Mean CSF vitamin B_{12} was 21 $\mu\mu$g./ml. In 17 cases CSF vitamin B_{12} equalled free vitamin B_{12} level in serum, in 16 cases CSF vitamin B_{12} was lower than the free level in serum, and in 10 cases CSF vitamin B_{12} was higher than the free vitamin level in serum. There was no apparent diagnostic correlation. The findings suggest that vitamin B_{12} is not bound in CSF and that there is some selective control of passage of vitamin B_{12} across the blood-CSF barrier.

ROSS[1] reported in 1950 that in the few cerebrospinal fluids (CSF) assayed for vitamin B_{12}, using *Euglena gracilis* as the test organism, he had found levels of 0-16 micro-micrograms/ml. ($\mu\mu$g./ml.). He also noted that heating CSF to 100° C. did not significantly increase the assay results; in the light of subsequent development of the technique this would indicate that B_{12} in CSF is in the free or uncombined form only.

Three other groups have studied vitamin B_{12} levels in CSF, but none reported the proportion of free to total B_{12}, or the relationship between CSF and serumfree vitamin levels. It was thought that such information might add to the available data on CSF dynamics, and possibly be of significance when extended to the group of demyelinating diseases.

In three papers published between 1958 and 1960, Sobotka and co-workers reported studies on CSF vitamin levels in normals and in patients with multiple sclerosis. Using *Ochromonas malhamensis* bioassay, Sobotka, Baker and Frank[2] found a normal range for B_{12} in CSF of 0-30 $\mu\mu$g./ml. The "normals" were 43 patients undergoing spinal anesthesia for minor surgical conditions. Concomitant serum B_{12} estimations ranged from 300 to 1000 $\mu\mu$g./ml. In 1958 Sobotka, Christoff and Baker[3] reported B_{12} and folic acid levels in CSF in 43 cases of multiple sclerosis. An elevation of CSF vitamin B_{12} was found in 14 of 41 patients. In 1960 Sobotka *et al.*[4] published a further study on CSF vitamin levels in multiple sclerosis. CSF vitamin B_{12} values were given for 41 cases (presumably the same 41 cases reported in 1958) and were elevated to 40-600 $\mu\mu$g./ml. in 14 of 41 patients. Only 21 of these 41 patients had serum B_{12} estimations. Six of the 21 had elevations of serum B_{12} ranging from 1100 to 800 $\mu\mu$g./ml. Correlation between the B_{12} increase in the sera and CSF was not good. Two of the patients with high serum levels had normal CSF values, one had a slight rise to 40 $\mu\mu$g./ml., two had gross elevations in both fluids, and with the highest CSF level of 600 $\mu\mu$g./ml. the serum B_{12} was only increased to 1100 $\mu\mu$g./ml. An additional 12 cases of multiple sclerosis had CSF vitamin B_{12} levels determined, and three of the 12 had values above normal, ranging from 40-80 $\mu\mu$g./ml. No serum B_{12} values were reported for these additional 12 cases. During the course of this multiple sclerosis investigation the vitamin B_{12} content of CSF was determined in 420 unselected neurological cases.

The authors stated that a relatively high percentage of the 420 cases had B_{12} values above the normal range, and that the incidence of high values seemed to be concentrated in patients with multiple sclerosis, but no details were given. In summary, Sobotka and co-workers claimed that the normal range for CSF vitamin B_{12} was 0-30 $\mu\mu$g./ml., and they found values above this normal in 17 of 53 patients with multiple sclerosis.

In 1961 Herbert and Zalusky[5] confirmed the observations of Sobotka's group for B_{12} levels in normal CSF. Using *Euglena gracilis* bioassay, they reported a mean CSF level of 22 $\mu\mu$g./ml., with an average serum value of 546 $\mu\mu$g./ml.; 14 subjects undergoing diagnostic study or spinal anesthesia were studied. They concluded that CSF appears to exclude protein-bound B_{12}, though no values for free and bound B_{12} were reported.

Using *E. coli* assay, Yavorkovsky and Mai[6] determined CSF vitamin B_{12} levels in 41 patients predominantly with neurological disorders. *E. coli* assay is not usually considered sensitive enough to detect B_{12} levels of less than 50 $\mu\mu$g./ml., though the authors claim that with their modification of the method results are reliable down to 20 $\mu\mu$g./ml. In 32 patients levels ranged from 0 to 15 $\mu\mu$g./ml., four were from 15 to 25 $\mu\mu$g./ml. and five patients had values between 51 and 65 $\mu\mu$g./ml. These authors reported a fairly good correlation between increased protein and increased B_{12} in the CSF. This occurred particularly with the meningitides,

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and the authors felt that in cases which did not show this correlation the reason might be that bacteria in CSF had utilized the B_{12}. They also felt that the increase of both protein and vitamin B_{12} in CSF was probably due to increased permeability of the vessels.

Reisner and Weiner[7] reported CSF levels of vitamin B_{12} in two cases before and after an intramuscular injection of 1000 μg. of B_{12}. Since they used *Lactobacillus leichmannii* as the test organism, and this organism is of low sensitivity and not entirely specific for B_{12},[8] their results are not very reliable.

The capacity of CSF to bind radioactive B_{12} (Co^{60}-B_{12}) was studied *in vitro* by Meyer, Bertcher and Mulzac.[9] They investigated 22 subjects with no apparent neurological disorder; specimens were obtained at the time of spinal anesthesia. These authors found that the serum-binding capacity was 2.5 to 3.5 higher than that of CSF, but that in relation to protein content, normal CSF had a higher binding capacity than normal serum. In both fluids the binding capacity increased as the concentration of radiovitamin was raised from 1 mμg. to 100 mμg./ml. of Co^{60}-B_{12}.

Using modern techniques, various workers have shown that CSF contains most of the proteins that have been identified in serum, though not necessarily in exactly the same proportion.[10, 11] Some of the CSF proteins show differences in physicochemical characteristics from their counterparts in serum.[12] The dynamics of CSF formation, circulation and reabsorption are still incompletely understood, but it is no longer possible to consider CSF a simple filtrate or transudate. Lowenthal, Karcher and van Sande[11] studied CSF and cerebral tissue proteins by both paper and agar electrophoresis. In some diseases, such as multiple myeloma, the same abnormal protein may be detected in serum, CSF and urine. Such parallelism has not been demonstrated in multiple sclerosis; on the contrary, abnormal CSF proteins do not have counterparts in the serum. In various encephalitides also, protein patterns in the serum differ markedly from CSF protein patterns. Lowenthal and co-workers have presented evidence to support the hypothesis that some abnormal protein components of CSF in disease originate within the central nervous system rather than in the serum.

In 1960 O'Connor *et al.*[13] reported that the mean serum B_{12} level in 21 patients with multiple sclerosis was 50% of the mean level in clinically healthy control subjects. *Lactobacillus leichmannii* was the test organism used for bioassay. They also stated that patients with multiple sclerosis had impaired ability to retain injected B_{12} in the tissues, although absorption was adequate. Grann and Glass[14] studied serum levels and intestinal absorption of vitamin B_{12} in multiple sclerosis. *Ochromonas malhamensis* was used for bioassay of the serum, and hepatic uptake of radioactive B_{12} for evaluation of intestinal absorption. They found that the majority of multiple sclerosis cases were normal in both respects. The findings of these two groups of investigators are considerably at variance with those of Sobotka's group, who reported a high incidence of high B_{12} levels in both serum and CSF in multiple sclerosis.

From the scattered reports in the literature briefly reviewed above, it seems that there is some ambiguity regarding CSF vitamin B_{12} levels, and the relationship between serum and CSF levels.

SUBJECTS

Owing to the low levels of B_{12}, relatively large amounts of CSF were needed for assay of free and total vitamin. It was seldom possible to obtain sufficient material from a routine spinal puncture, and cases had to be limited to those patients undergoing pneumoencephalography. Consequently, CSF was collected from cases with a variety of neurological diagnoses. Patients suspected to have infected CSF were not considered suitable for study; other than this there was no element of selection. The diagnoses listed in Table I were the final discharge diagnoses; it will be noted that two initially unsuspected encephalitides are included. No normal asymptomatic persons were studied, but in some of the patients no organic lesions were found. Five patients with demyelinating disease are included in the total group of 43 patients. None of the patients in the present series received B_{12} therapy in hospital before the specimens were obtained, nor was there any record of B_{12} therapy prior to admission.

METHOD

The biological method of Mollin and Ross,[8, 15, 16] using the Z strain of *Euglena gracilis*, was employed for the assays. The assay of B_{12} in CSF is essentially the same as the assay of B_{12} in serum. All glassware, pneumoencephalogram equipment, etc. in direct or indirect contact with the CSF specimens were subjected to special B_{12}-decontamination cleaning[17] before use. CSF specimens were centrifuged at 3000 revolutions per minute for five minutes immediately after collection to precipitate any cells, and the supernatant was stored at —20° C. until assayed.

Blood for serum B_{12} determinations was collected immediately before or after the pneumoencephalogram.

Two dilutions for free B_{12} and two for total B_{12} level were included in the first assay of the CSF. Dilutions of 1/8 and 1/16 were commonly used for both free and total assay, using duplicate tubes for each dilution. These dilutions occasionally had to be varied (1/4 to 1/40) in the repeat assay, depending on the values obtained in the initial assay. A 1/4 dilution is satisfactory for CSF assay owing to the low protein concentration in CSF, but in order to conserve material it was not used routinely. For complete B_{12} assay by this method 12-15 ml. of CSF is needed. Levels reported are the mean values for

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TABLE I.—LIST OF CASES, WITH DIAGNOSES AND LABORATORY FINDINGS

Case	Sex and Age	Final discharge diagnosis	Cerebrospinal fluid: RBC/c.mm.	WBC/c.mm.	% Polymorphs	% Lymphocytes	Protein mg.%	Colloidal gold curve	Kolmer	Other	Serum B_{12}* µµg./ml. Free	Serum B_{12}* µµg./ml. Total	CSF B_{12} µµg./ml. Free	CSF B_{12} µµg./ml. Total
GROUP 1. Cases with less than 10 µµg./ml. difference between free serum and CSF B_{12}.														
3	F 65	Amyotrophic lateral sclerosis	0	0	—	—	30	—	Neg.		24	242	11	15
4	M 41	Torticollis, traumatic sequela	0	1	—	—	40	—	NSQ		12	310	16	16
5	M 70	Arteriosclerosis of legs, heart and cerebellum	0	2	—	—	20	000000	Neg.	Chlorides 666 mg.%	7	306	6	7
8	M 54	Chronic brain syndrome, memory lapses	0	5	—	—	40	000111	Neg.		36	450	27	34
10	M 47	Cerebral contusion	1	0	—	—	40	—	Neg.		35	204	30	34
15	M 82	Cerebral arteriosclerosis	0	0	—	—	20	—	Neg.		2	56	<2	<4
19	M 23	Idiopathic epilepsy, fractured jaw	0	0	—	—	30	001100	Neg.	Chlorides 667 mg.%	12	383	14	17
22	M 21	Encephalitis (probably viral)	217	66	0	100	40	001210	Neg.		13	420	12	15
24	M 20	Idiopathic epilepsy	1	1	—	—	30	000100	—	Globulin—no increase	17	278	12	13
27	M 50	"Polyneuritis"	2	0	—	—	15	001210	—		3	339	10	10
29	F 37	Viral encephalitis (unlikely to be multiple sclerosis)	2	0	—	—	35	555543	NSQ		19	370	12	12
30	F 33	Tension headaches	3	1	—	—	20	—	—		10	200	12	14
31	M 32	Multiple sclerosis	2	13	16	84	75	444433	Neg.		5	185	5	8
32	F 69	Epilepsy, late onset-? arteriopathic	3	1	—	—	15	—	—		23	324	20	22
33	M 68	Neurosyphilis with vascular insufficiency	4	0	—	—	40	001222	Pos.		9	275	9	10
39	F 35	Multiple sclerosis, gastric ulcer	0	0	—	—	20	001111	NSQ		20	396	21	18
42	M 53	Chronic lead poisoning, acute arthritis right knee	0	2	—	—	30	001221	Neg.		38	448	28	34
Group 2. Cases in which free serum B_{12} was 10 or more than 10 µµg./ml. higher than CSF B_{12}														
6	F 35	Cerebral atrophy NYD, psychoneurosis	1	0	—	—	40	000110	Neg.	Pandy—slight increase	85	379	32	29
7	F 37	Migraine	0	0	—	—	20	—	—	Pandy—no increase	26	167	12	13
9	M 45	Temporal lobe epilepsy	0	0	—	—	35	—	Neg.		54	364	23	23
11	M 32	Psychoneurosis, tension state, stress erythropoiesis	0	8	0	100	20	000110	Neg.		43	326	28	28
13	F 37	Migraine headaches, anxiety state	0	0	—	—	20	—	—		34	243	22	23
14	M 53	Paraparesis, unknown etiology	2	7	—	—	30	000111	—	Globulin—no increase	37	402	22	24
16	M 34	Latent syphilis, rheumatic heart disease with mitral stenosis, chronic otitis media, anemia	0	0	—	—	NSQ	001100	Neg.	Chlorides 696 mg.% Sugar 53 mg.%	30	392	10	14
17	M 62	Parkinson's syndrome, cortical atrophy	2	0	—	—	40	—	—		51	451	21	23
20	F 47	Cortical atrophy, minor epilepsy	0	1	—	—	20	—	Neg.		114	385	22	23
25	F 38	Hyperostosis frontalis interna	3	16	21	79	30	000110	Neg.		75	281	29	30
26	M 13	Ataxia, cause undetermined	0	3	—	—	20	001110	—		32	436	16	21
34	M 45	Cerebral thrombosis, mild	0	0	—	—	15	—	Neg.		62	295	13	13
35	F 36	Syringomyelia, chronic anxiety state	4	2	—	—	30	—	Neg.		46	366	13	16
38	M 41	Toxic radiculoneuritis, Guillain-Barré syndrome**	0	1	—	—	150	442233	Prozoning	Sugar 105 mg.%	142	801	26	60
40	M 59	Cerebral atrophy, duodenal ulcer	2	3	—	—	20	000010	—		89	243	26	25
43	M 41	Ageusia, NYD	2	2	—	—	30	001210	Neg.	Globulin—no increase	55	500	18	17
Group 3. Cases in which CSF B_{12} was 10 or more than 10 µµg./ml. higher than free serum B_{12}														
1	F 62	Cerebellar ataxia (arteriosclerotic), osteoarthritis cervical spine	0	3	—	—	30	—	—		5	156	15	15
2	F 74	Focal seizures, left temporal area lesion	0	5	—	—	25	—	Neg.		9	355	17	20
12	F 64	Arteriosclerotic cerebrovascular disease, bronchiectasis	0	0	—	—	40	000110	Neg.		4	214	17	14
18	M 63	Cerebral contusion, alcoholism, subacute subdural	3	1	—	—	70	—	Neg.		10	350	33	23
21	F 47	Multiple sclerosis	3	0	—	—	30	444433	NSQ	Globulin—negative	24	238	38	38
23	F 79	Hypertensive cardiovascular disease, cerebrovascular accident	12	2	—	—	80	—	—		8	467	26	27
28	M 19	Suspect brain tumour, or low grade encephalitis or idiopathic epilepsy	6	28	12	88	20	001100	Neg.		8	507	25	26
36	F 17	Focal epileptic seizure NYD	8	5	—	—	40	000100	Neg.	Pandy—negative	13	471	23	24
37	F 37	Chronic anxiety state	0	0	—	—	20	001110	—		7	380	16	20
41	M 60	Astasia abasia, cervical and lumbar osteoarthritis, essential hypertension	377	0	—	—	30	000110	—	Globulin—no increase	12	349	34	38

*Normal serum B_{12} levels for this laboratory are: Free B_{12} = less than 100 µµg./ml.
Total B_{12} = 100 - 700 µµg./ml.
**Demyelination proved post mortem.

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the four assays (eight tubes), i.e. two initial and two recheck assays for both free and total B_{12}. Other details of the assay technique have already been published.[17]

Cell counts, protein estimations, colloidal gold curves, etc. were done in the routine hospital laboratory.

RESULTS

Free and total serum and CSF values for B_{12}, diagnoses and other pertinent data are presented in Table I.

Sera

In 40 of the 43 cases the serum levels for both free and total B_{12} were normal. The mean free serum level in these 40 cases was 28 $\mu\mu$g./ml., and the mean total level was 337 $\mu\mu$g./ml. Case 15 in Group 1 had low B_{12} levels in the serum, almost pathognomonic of pernicious anemia or subacute combined degeneration of the cord, but he had no physical signs or laboratory evidence of either condition. Case 38 in Group 2 had slightly increased serum levels of free and total B_{12}; this specimen was obtained one month before death and the increased values may be a reflection of beginning terminal liver dysfunction. Case 20 in Group 2 had a slight increase in free serum B_{12} with a normal total level.

Cerebrospinal Fluid

The free and total B_{12} levels in CSF were essentially the same in 42 of 43 cases. The measured differences ranged from 0 to 10 $\mu\mu$g./ml. with a mean value of 2 $\mu\mu$g./ml. Subsequent references to the level of B_{12} in CSF will mean free or total, whichever is the higher value. The details of CSF free and total levels are shown in Table I. The one exception was Case 38 in Group 2, in which the total level of B_{12} in CSF was double the free value.

The B_{12} level in CSF ranged from 0-50 $\mu\mu$g./ml. with a mean value of 21 $\mu\mu$g./ml. for 43 cases. Only one patient had no detectable B_{12} in CSF.

The cases in Table I are divided into three main groups, depending on whether the CSF levels were equal to, less than, or greater than the free B_{12} level in the serum.

Group 1 comprised 17 patients in whom there was less than 10 $\mu\mu$g./ml. difference between the free serum and CSF levels of B_{12}. The mean free level in the serum was 16.8 and in the CSF 16.6 $\mu\mu$g./ml. The measured values in the CSF in this group ranged from 0-34 $\mu\mu$g./ml.

Group 2 comprised 16 patients in whom the free B_{12} level in the serum was 10 or more than 10 $\mu\mu$g./ml. higher than the B_{12} level in CSF. The mean value for free B_{12} in serum was 61 $\mu\mu$g./ml. CSF level of B_{12} ranged from 13-50 $\mu\mu$g./ml., with a mean value of 24 $\mu\mu$g./ml. Expressed as percentages, CSF levels were from 20% to 68% of the free serum levels.

TABLE II.—DIAGNOSES AND FREE SERUM AND CSF LEVELS OF B_{12}

	Group 1	*Group 2*	*Group 3*
	B_{12} in CSF = free B_{12} in serum	*B_{12} in CSF less than free B_{12} in serum*	*B_{12} in CSF more than free B_{12} in serum*
Diagnosis	*No. of cases*	*No. of cases*	*No. of cases*
No organic lesion			
Cases 7, 11, 13, 30, 37	1	3	1
Arteriosclerosis			
Cases 1, 5, 12, 15, 17?, 40?	2	2	2
Epilepsy			
Cases 2, 9, 19, 20, 24, 32, 36	3	2	2
Demyelinating disease			
Cases 21, 27, 31, 38, 39	3	1	1
Infections			
Cases 22, 29	2		
Hemorrhage			
Cases 10, 18, 23, 34	1	1	2
Miscellaneous			
Cases 3, 4, 6, 8, 14, 16, 25, 26, 28, 33, 35, 41, 42, 43	5	7	2
Totals	17	16	10

Group 3 comprised 10 patients in whom the CSF level was 10 or more than 10 $\mu\mu$g./ml. higher than the free serum B_{12} level. The CSF values ranged from 15-38 $\mu\mu$g./ml., with a mean value of 26 $\mu\mu$g./ml. The mean free level in the serum was 10 $\mu\mu$g./ml.

CSF Protein and B_{12} Levels

The mean CSF protein level for 42 cases was 34 mg. %. Group 1 cases had a mean CSF protein level of 32 mg. % with a mean B_{12} level of 17 $\mu\mu$g./ml. Group 2 cases had a mean protein level of 35 mg. % with a mean B_{12} level of 24 $\mu\mu$g./ml. Group 3 cases had a mean protein level of 38.5 mg. % with a mean B_{12} level of 26 $\mu\mu$g./ml. It is doubtful if these differences are of any significance. Cases 18, 23, 31 and 38, which had above normal levels of CSF protein, had a mean B_{12} level in CSF of 30 $\mu\mu$g./ml.

Vitamin B_{12} Levels Related to Diagnoses

Table II subdivides the cases into broad diagnostic categories, and notes whether they are in Groups 1, 2 or 3 with reference to B_{12} serum and CSF correlation. There are too few cases to draw conclusions, but it would appear that there is no correlation.

DISCUSSION

Euglena gracilis is a very sensitive test organism; in our hands vitamin B_{12} in as little as 0.25 $\mu\mu$g./ml. can usually be detected, and 0.5 $\mu\mu$g./ml. regularly. Blank control tubes and standard tubes containing various dilutions of B_{12} are included in every assay batch. The six control tubes usually have a mean optical density reading between 3 and 4. The weakest dilution of B_{12} included in the standard tubes is 0.25 $\mu\mu$g./ml., and the mean optical density readings for the six tubes of this dilution range

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from 5.5 to 6.5. These mean values have always shown good differentiation; very rarely one of the 0.25 $\mu\mu$g./ml. standard tubes may be as low as the highest blank control tube. Ross[15] states that with the Euglena test organism as little as 1 $\mu\mu$g./ml. of B_{12} can be detected, but that in serum it is not practicable to measure less than 10 $\mu\mu$g./ml., as serum present in greater concentration than 1 in 8 coagulates with heating to 100° C. and may be inhibitory. This limitation should not apply to free serum assays, in which the tubes are only heated to 56° C. Ross states also that CSF can be set up for assay in 1/4 concentration because of its low protein content. Although the 10 $\mu\mu$g./ml. limit of sensitivity probably does not apply to free serum assays or CSF assays, we selected the 10 $\mu\mu$g./ml. difference between free serum and CSF vitamin B_{12} levels to subdivide our cases, as this is the generally accepted level of sensitivity.

The overall mean level of 21 $\mu\mu$g./ml. of B_{12} in CSF in the present miscellaneous group of patients is almost identical with the mean of 22 $\mu\mu$g./ml reported by Herbert and Zalusky[5] for normal individuals. Only eight of the present series of 43 patients had CSF levels of B_{12} above the normal range of 0-30 $\mu\mu$g./ml. determined by Sobotka and co-workers;[2] only one of five patients with demyelinating disease was above 30 $\mu\mu$g./ml.

The present authors are unable to confirm Sobotka and co-workers' report[4] of high serum and CSF levels of B_{12} in multiple sclerosis, or O'Connor and co-workers'[13] findings of low serum B_{12} values in this disease. The number of patients with demyelinating disease in the present series is small, but seven of eight above normal levels of CSF B_{12} were in patients who did not have demyelinating disease. Sobotka and co-workers' values, for both serum and CSF levels of B_{12}, appear excessively high when compared with the results of other investigators, yet they were using a test organism considered to be the most specific.[8] It is not stated whether any of their patients were receiving B_{12} therapy, or had done so in the recent past.

In normal serum, B_{12} is bound to alpha-globulin, though other serum globulins are involved when serum B_{12} levels are increased in some disease states. Since high molecular weight α_2-macroglobulins have been detected in CSF,[12] one cannot postulate that serum-bound B_{12} is too large a particle to pass the blood-brain or blood-CSF barrier. Lowenthal and his group[11] did not report finding any macroglobulins in cerebral tissue. Free B_{12}, with a molecular weight of approximately 1360, should be able to circulate freely between serum and CSF, but free serum and CSF levels cannot be correlated. If, as reported by Meyer's group,[9] the binding capacity of CSF in relation to protein content is greater than the binding capacity of serum, one would expect that total B_{12} in CSF would be higher than free B_{12}, but only in Case 38 was there any evidence of B_{12} binding in CSF. The results of this study suggest that (a) there is no bound B_{12} in CSF in the majority of cases and (b) there must be some selective control of B_{12} passage at the blood-CSF barrier.

For the 10 cases in Group 3, in which the B_{12} level in the CSF was higher than the free level in the serum, no reasonable explanation was discernible. Two of the 10 cases had increased CSF protein, but so did two cases in the other groups.

While the measured differences between free B_{12} in serum and B_{12} in CSF may be arithmetically significant, in the majority of cases (28, or 65%) the difference was less than 16 $\mu\mu$g./ml. and is not likely of any biological significance. In a further seven cases (16%) the difference was less than 31 $\mu\mu$g./ml., of doubtful biological significance. The remaining eight cases (19%), all from Group 2 with higher free levels in the serum, had differences ranging from 31-92 $\mu\mu$g./ml., probably biologically significant. On the other hand, there are no data on what is the minimal requirement, if any, of B_{12} in the CSF.

SUMMARY AND CONCLUSIONS

Free and total levels of vitamin B_{12} were assayed in serum and cerebrospinal fluid (CSF) in 43 patients from the neurological service. CSF levels of vitamin B_{12} ranged from 0-50 $\mu\mu$g./ml. with a mean value of 21 $\mu\mu$g./ml. Only one patient had no detectable B_{12} in CSF. Free and total levels of vitamin B_{12} in CSF were the same in 42 patients. In 40% of the cases the vitamin B_{12} level in CSF was the same as the free level in the serum, in 37% the CSF level was lower than the free level in the serum, and in the remaining 23% the CSF level was higher than the free level in serum. There were three patients with multiple sclerosis. In two the serum and CSF levels were normal; the third case had a slight elevation of vitamin B_{12} in the CSF though serum levels were normal. Of two other patients with demyelinating disease, one had normal serum and CSF levels, and the other had slight elevation of free and total serum vitamin B_{12} and a total CSF level double the free level, suggesting some binding of vitamin B_{12} in CSF.

The findings suggest that there is some selective control of passage of vitamin B_{12} across the blood-CSF barrier. While the measured differences between free serum and CSF vitamin B_{12} may be arithmetically significant, a biological significance is considered possible in only 19%. In this limited number of cases no diagnostic correlation was apparent.

We gratefully acknowledge the co-operation of Dr. C. E. G. Gould and the section of neurology, without which this study could not have been made. We wish to thank Mr. Michael Dane, of the Department of Slavonic Studies, University of British Columbia, for the translation of Yavorkovsky and Mai's paper.

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The Serum Vitamin B_{12} Concentration in the General Population

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The serum vitamin B_{12} concentration was estimated in a random sample of two communities; an industrial and an agricultural one. A total of 890 people were studied. The frequency distribution of serum vitamin B_{12} concentration shows that the range of values is wide and that the serum vitamin B_{12} level falls with increasing age. For general purposes the results of this study may be summarised by standardising at age 50 years, using the regression coefficient -1.254 $\mu\mu$g per ml per year of age, which summarises the gradient with age over the whole age range studied. Thus the adjusted means and standard errors, 255.2 ± 5.45 $\mu\mu$g per ml for the industrial men and 262 ± 10.06 $\mu\mu$g per ml for the women, compare closely with the agricultural community where the figures for the men are 260 ± 9.52 and for the women 272 ± 8.45 $\mu\mu$g per ml.

During studies made on the distribution of various haematological indices and on the prevalence of anaemia in random samples of an industrial (mining) and an agricultural community in the United Kingdom, sufficient blood was obtained from the majority of subjects for the concentration of serum vitamin B_{12} to be determined. Details of the original findings have been published (Kilpatrick & Hardisty 1961, Kilpatrick 1961) and we now report on the distribution of serum vitamin B_{12} concentration in these communities.

POPULATION SAMPLES

Details of the methods of drawing the population samples have been given in the earlier publications and the numbers of subjects examined in this study are shown in Tables I and II. For various reasons is was not possible in every instance to obtain sufficient blood for serum vitamin B_{12} estimation but we feel that a sufficiently high number of subjects was examined to make the studies useful.

LABORATORY METHODS

Serum was separated from the clotted specimen of blood as soon as it reached the laboratory and was stored at $-20°$ C until assayed. The technique of assay was modified from that described by Ross (1952) using Euglena gracilis Z strain (Hutner *et al.* 1956).

TABLE I

Population samples – Industrial population – Rhondda Fach

Sex/occupation group	Age	No. in sample	Examined	B_{12} estimated
	35–44	100	90	87
Miners and ex-miners	45–54	100	95	88
	55–64	100	93	83
	35–44	100	85	82
Non miners	45–54	100	89	84
	55–64	100	91	85
Women	55–64	200	180	164
		800	723	673 (84 per cent) of total sample

TABLE II

Population samples – Rural population – Wensleydale

Sex	Age	No. in sample	Examined	B_{12} estimated
Men	15–24	53	29	27
	25–34	37	26	25
	35–44	54	34	31
	45–54	48	36	33
	55–64	33	25	24
	65–74	27	20	18
	75+	13	12	12
		265	182	170 (64 per cent)
Women	15–24	45	32	30
	25–34	51	40	39
	35–44	52	45	43
	45–54	42	34	33
	55–64	38	32	31
	65–74	37	33	32
	75+	17	14	9
		282	230	217 (77 per cent)

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Tryptone water was omitted from the stock culture medium as it seemed to favour the growth of yeasts. The organism for inoculation was kept on solid agar medium consisting of basal medium, tryptone and 2 per cent agar. From this agar slope a tube of single strength medium containing 15 $\mu\mu g$ of vitamin B_{12} per ml was inoculated and used the following week as inoculum for the test. Achlorophyllic varieties of Euglena were not so likely to arise when weekly sub-cultures were made from a solid medium as they were from liquid.

The sera were first set up in four tubes at dilutions of 1/40 and four at 1/80. Some sera were subsequently set up at dilutions of more than 1/80, as indicated, for the second assay. The majority of the sera was assayed twice and sometimes three times when the quantity of sera was sufficient, but in a few cases there was only enough serum for a single assay.

The vitamin B_{12} standards were originally modified from those of Ross (1952) to the following: 0, 1.25, 2.5, 5.0, 7.5, 10 and 15 $\mu\mu g$ per ml. It was found that the growth curve rose too steeply at concentrations above 15 $\mu\mu g$ per ml for readings to be accurate. The standard concentrations were subsequently changed to 0, 1, 2, 4, 6, 8, 10, 12 and 14 $\mu\mu g$ per ml.

RESULTS

Figures 1–3 show frequency distributions of serum vitamin B_{12} concentrations in the different age, sex and occupation groups examined, together with the mean value for each group. In the mining community (the Rhondda) there was no difference between miners and ex-miners taken together and non-miners, the figures have therefore been combined for subsequent analysis. It should be noted that in this study a limited number of age groups were examined.

In the agricultural community (Wensleydale) the mean values of serum vitamin B_{12} concentration for men are on the average slightly lower than those of the women, and diminish with age in men of 55 and over. In the same age groups the levels in the Rhondda and in Wensleydale were very similar.

Considering the two samples together the results may most conveniently be summarised by standardising at age 50 years using the regression coefficient — 1.254 $\mu\mu g$ per ml per year of age, which summarises the gradient with age over the whole age range studied (though as has been said the change is really confined to the older ages).

Adjusted mean and standard error (B_{12} $\mu\mu g$ per ml)

	Men	Women
Rhondda	255.2 ± 5.45	262 ± 10.06
Wensleydale	260.0 ± 9.52	272 ± 8.45

The results are not significantly different one from another. The only finding of statistical significance is the overall decline with age as measured by a linear regression coefficient.

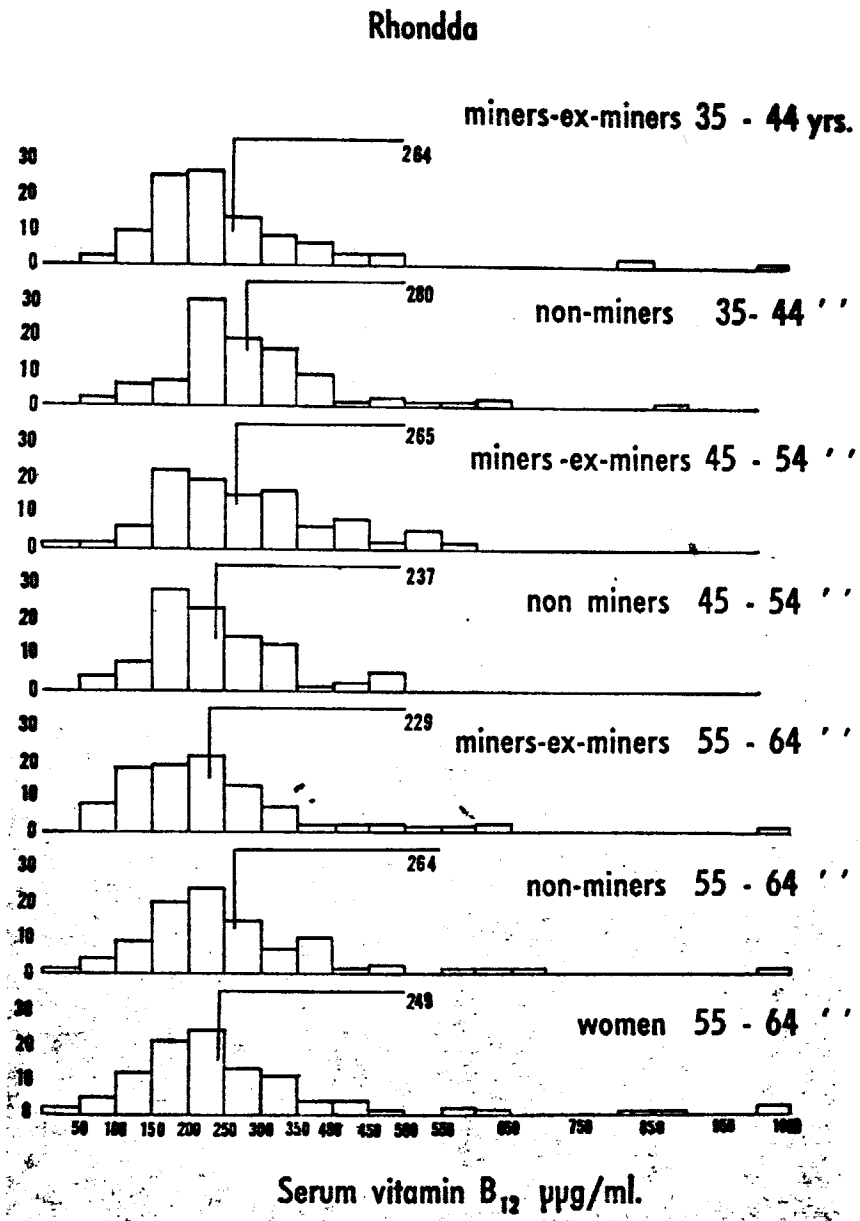

Figure 1. Frequency distribution of serum vitamin B_{12} concentrations in Rhondda population. – The mean value is shown for each group.

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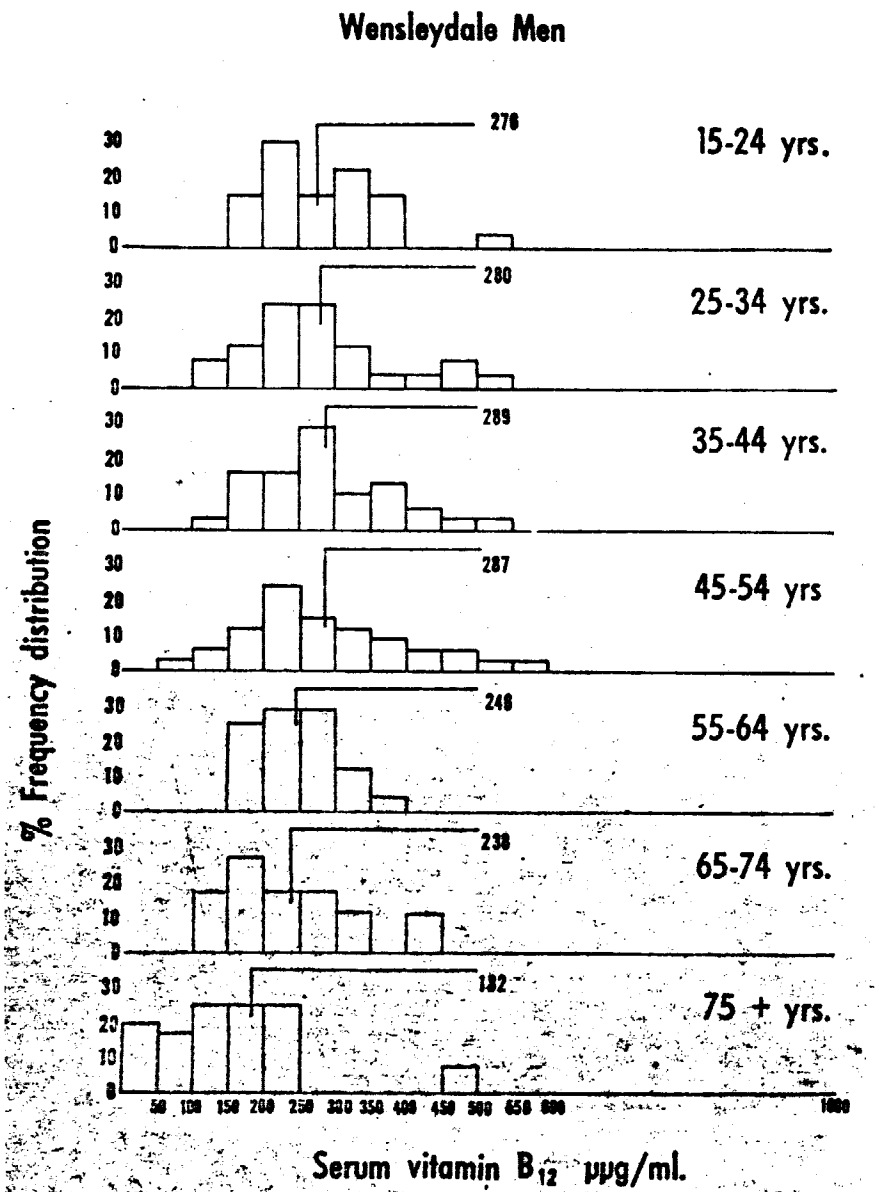

Figure 2. Frequency distribution of serum vitamin B_{12} concentrations in Wensleydale men. – The mean value is shown for each group.

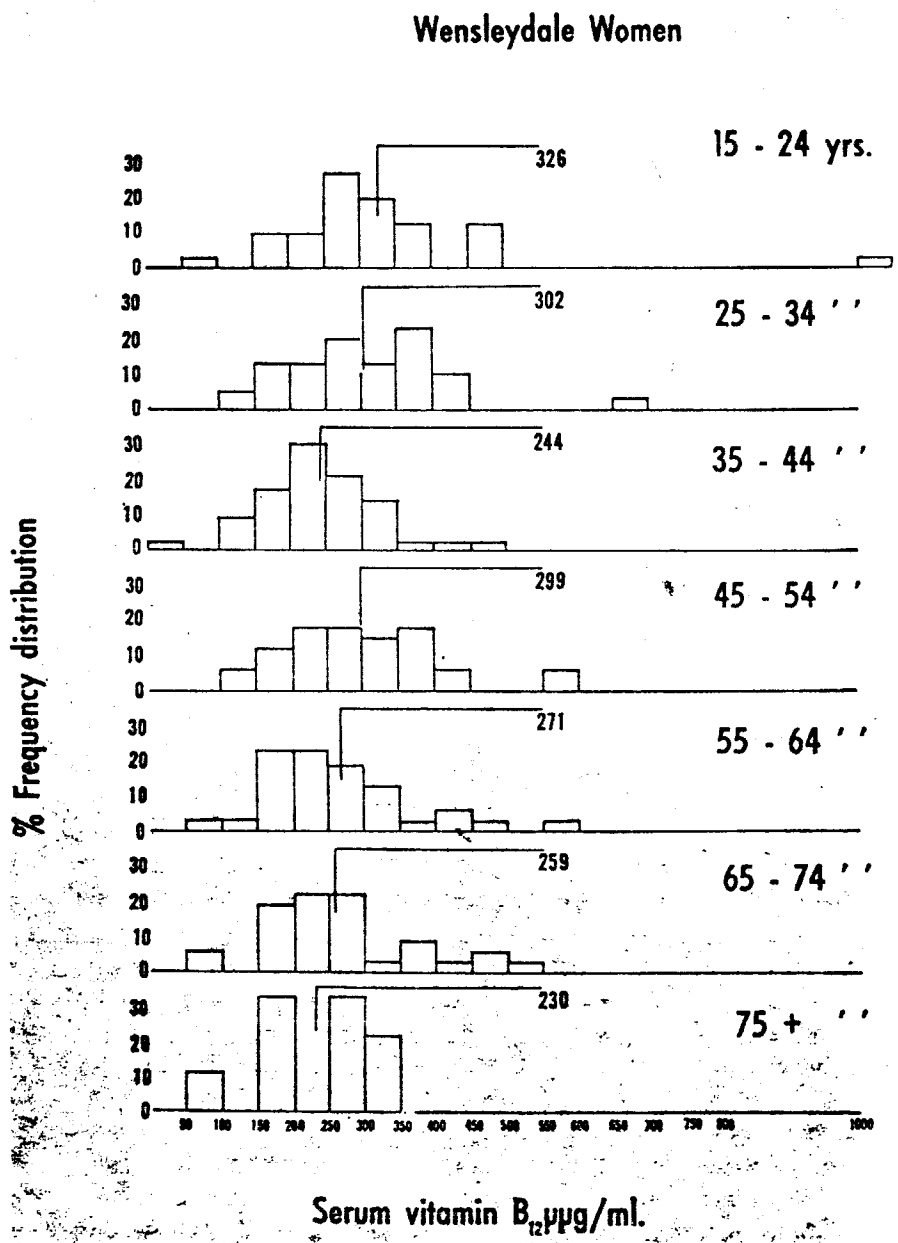

Figure 3. Frequency distribution of serum vitamin B_{12} concentrations in Wensleydale women. – The mean value is shown for each group.

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Tests were made to determine whether there was any correlation between the serum vitamin B_{12} and the serum iron concentrations because certain inter-relationships have been reported in disease (Cox *et al.* 1959). The average correlation in the sample examined was only -0.021 and when this was corrected for the correlation with age it became -0.033: very small and not significantly different from zero. While this was true of the Rhondda miners, ex-miners, non-miners and the Wensleydale men, there was a correlation of -0.193 between serum iron and serum B_{12} in the Wensleydale women the explanation of which is not apparent; thus on average, in this group of women, the serum iron diminished by 0.107 μg per 100 ml for every unit increase of serum vitamin B_{12}.

FOLLOW UP STUDY

To try to find out the importance of unusually low and unusually high concentrations of serum vitamin B_{12} concentrations we followed up, for 3 years, 43 persons all of whom had concentrations of 100 $\mu\mu$g per ml or less or 800 $\mu\mu$g per ml or more. For geographical reasons this study could only be done in the Rhondda.

Thirty six of the original 43 people were seen again. The subjects were visited at home, interviewed, and a specimen of blood was taken for haematological investigation and repeat B_{12} estimation. Further investigation was then offered to those people in whom the blood was abnormal.

Of the original 10 subjects who had a serum vitamin B_{12} concentration of *800 $\mu\mu$g per ml or more* 9 were seen again and in each case the new figures were within the accepted normal range though at the upper end of it. The high figures found originally may partly be explained by the fact that in several instances no replicate measurements were made but Mollin & Ross (1952) have shown that abnormally high concentrations of B_{12} may be due to unusually large amounts of free vitamin temporarily in the serum. Unfortunately we were not able to determine whether this was so in our cases.

Of the original 33 subjects who had a serum vitamin B_{12} concentration of *100 $\mu\mu$g per ml or less*, 27 were seen subsequently. In the majority (23) the concentrations were very similar to those found before. In 3 however, the concentrations were lower and one man who came for further investigations was shown to have pernicious anaemia. One woman whose concentrations was now much higher had been discovered to have pernicious anaemia in the interval between the two studies and had been given injections of vitamin B_{12}.

Thus in the first survey one subject was found to have pernicious anaemia and over the succeeding years two others developed it both of whom had a low (but not very low) serum vitamin B_{12} at the time of the original survey.

DISCUSSION

Despite the frequency with which the concentration of vitamin B_{12} in the serum is estimated today, surprisingly few studies have been made on representative samples of the general population. Our findings in this random sample of the general population confirm and put on a more secure footing the findings of the majority of other workers whose subjects were largely drawn from selected populations.

Mollin & Ross (1952) examined three age groups 15–40 (65 subjects), 51–70 (27 subjects) and 71–93 (31 subjects) – sex not designated – using the Euglena gracilis technique and found that the last age group showed a significantly lower mean value than the first two. Boger *et al.* (1955) examined 528 people in the United States of America – blood donors, members of State Institutions and schools – using the L. leichmanni method and found a trend towards a lower serum concentration of vitamin B_{12} with increasing age. They did not find any difference between the sexes. Chow *et al.* (1956), again in the United States, examined 324 subjects – students and immates of a penal institution. Using L. leichmanni they also found higher concentrations in the young than in the old. Thus the prisoners with an average of 23.3 years had a mean B_{12} concentration of 268.2 ± 16.5 $\mu\mu$g per ml and those over the age of 66 years 180.5 ± 25.5 $\mu\mu$g per ml. Gaffney *et al.* (1957) studied various groups of people in Baltimore and again found a gradual decline in serum vitamin B_{12} concentration with advancing age but no correlation with diet or socio-economic factors. Kristensen (1956) on the other hand, using L. leichmanni organisms found no difference between 55 subjects aged 20–40 years and 29 subjects aged 67–85. Similarly Killander (1957) in Sweden using Euglena gracilis var. bacillaris examined 165 adults between 15 and 82 years and found the highest mean value occurred below the age of 19 years. After this age he could not detect any fall with age, though he reported that women over the age of 20 had a slightly lower mean value than men.

While some workers have not found any correlation between serum vitamin B_{12} and diet when the diet was normal or near normal, Banerjee (1960) showed that in vegetarians, a low level of serum vitamin B_{12} could be demonstrated which he ascribed to an inadequate dietary intake. Smith (1962) reported on 12 vegans (strict vegetarians) who had an average serum vitamin B_{12} concentration of 128 ± 15 $\mu\mu$g per ml. Although few of these patients

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had symptoms two had early subacute combined degeneration of the spinal cord. The serum B_{12} concentration in pregnant women tends to be low, Nesbitt & Chow (1958), possibly because the requirements are increased but we have no data on this.

From our own studies and the work of others it is evident that the serum B_{12} concentration tends to be high in young people and low in the elderly. It may be that there is impaired absorption of vitamin B_{12} in the elderly because old people retain a higher proportion of an injected dose of B_{12} (Tauber *et al.* 1957) than the young. It should be remembered however, that Spray (1962) has demonstrated that the serum concentrations of vitamin B_{12} do not necessarily reflect the total body stores of the vitamin.

Our follow up study shows that abnormally high values may occur occasionally without apparent reason; they are probably not important. People with serum vitamin B_{12} concentration of 100 $\mu\mu$g per ml or less may come into the groups of pre or latent pernicious anaemia described by Robertson *et al.* (1955) and Wood *et al.* (1960).

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Relationship of Vitamin B12 and Folic Acid to Memory Function

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Sixty-three aged subjects, most of them suffering from senile and/or ateriosclerotic brain disease, were investigated psychiatrically and neurologically, and tested psychometrically. Their serum cyanocobolamine (vitamin B12) and folic acid level was determined. The data obtained were analyzed statistically. Vitamin B12 and folic acid deficiency were found in a high percentage of all subgroups including the well-preserved subjects. No significant correlation was found between vitamin B12, or folic acid, or combined deficiency, and the measures of senile memory impairment (memory quotient and retention span). However, in the group with an amnestic syndrome, the B12-to-folic acid ratio decreased with decreasing MQ (memory quotient) and retention span; that is, the serum B12 decreased whereas the folic acid level increased. A therapeutic trial, whereby 18 subjects with senile or arteriosclerotic memory defect received 7000 μg of vitamin B12 within a 3½-months period, did not lead to any improvement of memory function as compared with an untreated matched control group.

INTRODUCTION

Recent experimental evidence seems to indicate that brain ribonucleic acid (RNA) is somehow related to memory function. This has led to an active search for substances which might improve memory function and learning by influencing the RNA concentration of the brain (Cameron *et al.*, 1966; Glasky and Simon, 1966). Vitamin B12 (cyanocobolamine might be such a substance. It is necessary for the methylation of transfer RNA, thus controlling the rate at which protein synthesis takes place (Venkataraman *et al.*, 1967). In addition, vitamin B12 administration has been shown to increase the RNA concentration of neurons (Alexander and Backler, 1951; Enesco, 1967), and to increase the performance and learning ability of rats (Corson and Enesco, 1968).

On the other hand, the possibility that vitamin B12 deficiency might be associated with memory impairment in the aged had previously been suggested in the literature. It is known to induce neurological damage (Holmes, 1956), and has been reported to occur with greater frequency in psychiatric patients than in a normal population (Edwin *et al.*, 1965; Shulman,

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limit of the retention span; (iii) upper limit of the retention span; and (iv) weight. This analysis was carried out for the group as a whole, and for the subgroups of subjects with B12 deficiency, folate deficiency, and combined deficiency.

RESULTS

Vitamin B12 and folic acid deficiency were present in a surprisingly high percentage of subjects. Eleven out of 53 subjects, i.e., 28%, where MQ could be determined, had a serum B12 level below 200 μμmg/ml; while 16, i.e., 36%, were deficient in folic acid, that is, their serum folate level was lower than 4 μmg/ml; six subjects, i.e., 11%, of the total group of 53, were deficient in both substances.

However, B12 and folate deficiency were found not only in patients with impaired memory function. Figure 1, where the population is grouped according to the degree of memory impairment in four groups (MQ below 60, 60–80, 80–100, and above 100) shows that B12 as well as folate deficiency is found in a fairly high percentage in each group. For instance, in the group with MQ of 100 and over, comprising 17 subjects, seven, i.e., 39%, had B12 deficiency, and the same number folate deficiency (Fig. 2).

The correlation study did not reveal any significant correlation between the level of serum vitamin B12 or folic acid and any measure of senile memory impairment in the population as a whole. If, however, the same analysis was performed separately for each sex, it was found that in women the serum vitamin B12 level was positively correlated with MQ at the 0.1 level of significance.

A summary of the data is presented in Table II, where patients are grouped according to MQ level. Although the table shows that only the subjects with MQ below 60 have an average B12 level below 200 μμmg/ml, an interesting trend appears when the B12 and folate values in the three subgroups with MQ below 100 are compared: the lower the MQ, the lower the average serum vitamin B12 level and the higher the average serum folate level. In the patients with MQ below 60, the serum vitamin B12 is only 153 μμmg/ml, but it rises to 304 and 409 μμmg/ml respectively as MQ increases. The average serum folate level, on the other hand,

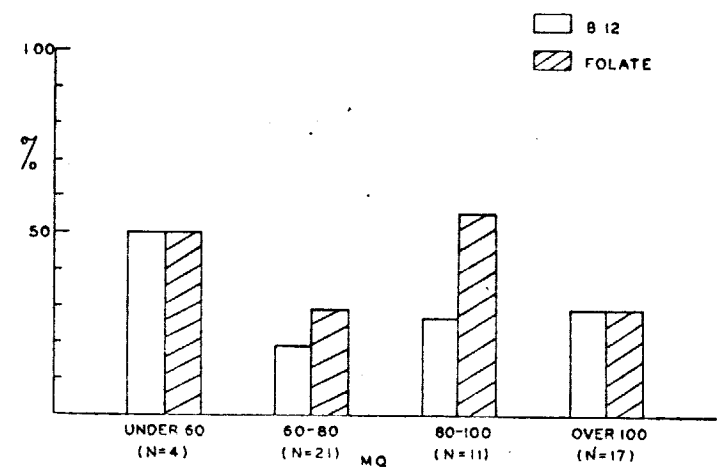

Fig. 1. Percentage of patients with B12 or folate deficiency grouped according to memory quotient.

1967). While some investigators reported that serum vitamin B12 levels decrease in aging (Chow *et al.*, 1957; Gaffney *et al.*, 1957), others found normal values in advanced-age groups (Shulman, 1967; Kirk, 1962). Droller and Dosset (1959) reported that aged individuals with senile dementia have a significantly lower serum vitamin B12 level than normal persons of a similar age, although no quantitative measures of memory impairment were taken in their study. Dawson and Donald (1966) reported that low serum vitamin B12 levels were correlated with both age and mental symptoms; 26% of their patients, 70 years and over, with low serum vitamin B12 levels, were clinically either confused or demented.

Folic acid also participates in nucleic acid metabolism (Weissbach and Dickerman, 1965; Robinson, 1966), and folic acid deficiency has been observed in psychiatric patients with a reported frequency of 50–80% (Carney, 1967; Hunter *et al.*, 1967), particularly in patients with organic brain disease (Reynolds, 1967). On the other hand, no difference was found in the serum folic acid levels between young and aged persons living at home (Reizenstein, 1965; Girdwood *et al.*, 1967) while low serum folate values have been reported for hospitalized geriatric patients (Hurdle, 1966). However, no studies on memory function in relation to serum folic acid level seem to have been carried out by previous investigators. Because of these somewhat conflicting reports, we tried to determine whether serum vitamin B12 and folic acid levels are correlated with the degree of memory impairment present in the aged, and whether parenteral administration of vitamin B12 would improve their memory function. It is the purpose of this presentation to report on our findings.

MATERIAL AND METHOD

Part 1

A total of 63 subjects (37 men and 26 women) was selected from the geriatric wards of two hospitals and from two homes for the aged in the Montreal area. The mean age of these subjects was 77 years, and the age range extended from 51 to 92 years. Table I shows the distribution of these subjects according to diagnosis, age, and sex.

The number of subjects is relatively small because patients suffering from subacute combined degeneration of the cord, and those with sensory deficiencies or aphasia who could not participate in psychological testing, had to be excluded from the total number of cases originally at our disposal. Patients on folic acid, chlorpromazine, or anticonvulsive agents, as well as those who had received antibiotics, chemotherapy, or blood transfusions within two weeks, or vitamin treatment within three months prior to the study, were also excluded.

Table I. Distribution of Subjects According to Diagnosis, Age, and Sex

Group	Males	Females	Total	Age Range	Age Mean
Without psychological or neurological symptoms	12	0	12	51–81	73
Senile dementia	3	6	9	66–90	78
Cerebral arteriosclerosis	9	15	24	62–89	78
Diagnosis undetermined	13	5	18	67–92	77
Total	37	26	63	51–92	77

All subjects were examined psychiatrically and neurologically. Memory function was measured by the standard Wechsler Memory Scale (Wechsler, 1945). In addition, the counting test (Cameron, 1943) was used to determine the upper and lower limit of the retention span. The neurological condition was rated by a scoring system, details of which will be published separately. Perlin and Butler's (1953) age-relevant check list was used for the evaluation of the type and degree of dementia present. Finally, a total deterioration score was computed for every subject using the age, MQ, the lower limit of the counting test, the neurological score, and the total score of the age-relevant check list.

Blood samples were taken for determination of serum vitamin B12 and folate levels within 48 hr after completion of the memory tests. Serum vitamin B12 level was measured by biological assay according to the method of Ross (1952) using *Euglena gracilis*. Values below 200 $\mu\mu$mg/ml were regarded as evidence of vitamin B12 deficiency. If vitamin B12 deficiency was found, further investigation—where practical—was carried out. Serum folic acid was determined by the method of Baker *et al.* (1959) using *Lactobacillus casei* in a microbiological assay system. Values below 4 μmg/ml were considered evidence of folic acid deficiency.

Analysis of Data

A computer was used to study all possible correlations among the following variables: age; weight; Memory Quotient; lower limit of retention span; upper limit of retention span; neurological test score; clinical diagnosis of cerebral arteriosclerosis or senile dementia; total score on the age-relevant check list; scores on questions relevant to cerebral arteriosclerosis or senile dementia on the age-relevant check list; and total deterioration score.

These correlations were determined separately for the total group, the males, the females, the subjects with MQ less than 100, and those with MQ of 100 and over.

The average serum vitamin B12 and folic acid values were calculated for subjects whose MQ was higher than 100, 100–80, 80–60 and under 60. Finally, a *t*-test was applied to discover any significant difference between the groups.

Part 2

The second half of the investigation was a single-blind study of the effect of vitamin B12 treatment on patients with senile memory impairment. Eighteen subjects (7 men and 11 women) formed the experimental group, and 22 (12 men and 10 women) the control group. They were matched according to the institution where they were treated, age, MQ, diagnostic category, vitamin B12, and folic acid level.

The experimental group received 100 μg vitamin B12[2] im 5 times weekly for 14 weeks; the control group remained untreated. At the end of the 14 weeks, the subjects were retested on the Wechsler Memory Scale and the Counting Test. Weights were also recorded, both before and after the treatment period.

Analysis of Data

The data were analyzed by a two-way analysis of variance performed on the values obtained before and after treatment. The four variables analyzed were: (i) MQ; (ii) lower

[2] Trademark Rubramin, courtesy of the Squibb Company, Montreal.

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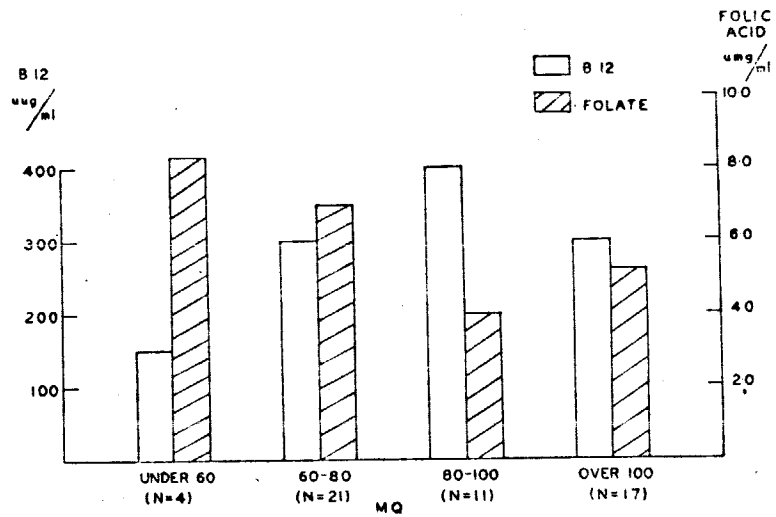

Fig. 2. Average serum B12 and folic acid values in patients grouped according to memory quotient.

decreases from 8.3 to 6.9 μmg/ml as MQ increases. Consequently, the serum B12-to-folate ratio is low in the subjects with MQ below 60, namely, 1:18, and increases with increasing MQ.

To determine whether this trend was statistically significant, correlation analyses were performed. It was found that the B12-to-folate ratio did not correlate significantly with MQ, but did correlate positively at the 0.01 level of significance with the senile score on the age-relevant check list.

Table III summarizes the results of the therapeutic trial. No significant difference in the mean memory quotient was found between the experimental and the control group as a result of 14-weeks vitamin B12 treatment, by which time each patient in the experimental group had received 7000 μg of B12.

Table II. Subjects Grouped According to Memory Quotient

	Over 100	80–100	60–80	Under 60
Number of subjects	17	11	21	4
Mean age	75	80	78	69
Age range	51–88	67–92	70–88	62–76
Average MQ	110	89	71	57
Retention span:				
Lower limit	69	47	21	17
Upper limit	95	61	31	27
Average serum B12 $\mu\mu$mg/ml	294	409	304	153
Average serum folate μmg/ml	5.20	4.00	6.96	8.30
Ratio Folate/B12	1:56	1:100	1:42	1:18

Table III

Group	Number		Age		Weight		MQ				Lower limit of retention span				Upper limit of retention span			
	Exper.	Cont.	Exper.	Cont.	Exper.	Cont.	Exper. Bef	Exper. Aft	Control Bef	Control Aft	Exper. Bef	Exper. Aft	Control Bef	Control Aft	Exper. Bef	Exper. Aft	Control Bef	Control Aft
B12 deficient (with or without folate deficiency)	3	6	81	79	135	128	93	75	94	94	62	27	57	26	88	61	63	78
Folate deficient (with or without B12 deficiency)	7	6	79	76	103	MD[b]	93	85	90	89	41	33	53	32	64	56	73	75
Combined deficiency	2	3	78	74	MD[b]	MD[b]	107[a]	80[a]	85[a]	88[a]	75	22	54	27	93	50	58	81
No deficiency	10	13	78	78	123	126	95	95	81	89	36	20	34	22	46	47	46	55
Total group	18	22	79	78	127	128	90	87	88	92	38	26	45	27	48	42	77	72

[a] Treatment difference.
[b] Missing data.

Furthermore, no significant change of the MQ was observed in either the vitamin B12 deficient subgroup, or in the subgroup with folic acid deficiency, as a result of B12 treatment. In the subgroup with combined deficiency, MQ of the two control subjects increased minimally, whereas the mean MQ of the two experimental subjects dropped significantly after treatment. This was probably due to a stroke which one of the patients in this experimental subgroup suffered during the treatment period. There was no significant difference in the means of the upper or lower limits of the retention span between experimental and control subjects, neither in the total group, nor in any of the subgroups. It should be noted also that prolonged parenteral administration of vitamin B12 did not significantly increase the weight of the experimental group as compared to the control group, nor did the weight change in the vitamin deficient subgroup as compared to that of the control group.

DISCUSSION

Contrary to some reports, our investigation could not demonstrate any clear-cut correlation between measures of senile deterioration as used in this study and the serum vitamin B12 or folic acid levels. These values *per se* were not correlated with the degree of memory impairment, or the presence and severity of the neurological symptomatology in our patients. It should be remembered that not even in pernicious anemia, where B12 and folate deficiency play an important role, did the neurological or psychiatric symptomatology, if present at all, strictly parallel the hematological findings. Similarly, mental symptoms in pathological old-age, particularly memory impairment, may not parallel vitamin B12 and folic acid deficiency. However, our results do not indicate that vitamin B12 or folic acid deficiency are more frequent in senility than in normal senescence. Rather, they seem to indicate that a relationship exists between the serum B12 and folic acid ratio on the one hand, and the degree of senile memory impairment on the other hand. The subgroup analysis showed that with decreasing MQ and retention span, the average B12 level in the serum decreases, whereas the average folic acid level increases. That this decrease in the serum vitamin B12-to-folic acid ratio was found only in the subgroups with quantitatively measurable memory deficit may be due to the fact that, in our material, the subgroup with MQ of 100 and over consisted largely of well-preserved aged people whose memory impairment, if any, was of the "benign" type, whereas the subjects of the subgroups with the lower MQ showed qualitatively the "malignant" type of memory impairment, that is, an amnestic syndrome due to senile or arteriosclerotic brain disease (Kral, 1966).

It is known that folic acid treatment of pernicious anemia may precipitate or aggravate neurological symptoms. It is possible that in such cases the administration of exogenous folic acid upsets the balance between the serum vitamin B12 and folic acid level. One may speculate that in our material with senile and/or arteriosclerotic brain disease, an increase in serum folic acid level in the presence of subnormal or even normal vitamin B12 levels may upset a precarious balance in the metabolic processes necessary for brain protein synthesis and nucleic acid metabolism.

On the basis of this assumption, it is not surprising that the therapeutic trial with vitamin B12 had no effect on the memory scores of our patients, even if they were deficient in vitamin B12, or folic acid, or both. Rather, the negative results underline the conclusions derived from the first part of our study. Since vitamin B12 deficiency *per se* does not seem to be the cause of the memory impairment in the senile individual, the administration of vitamin B12 alone cannot be expected to improve memory function. Whether the administration of substances capable of reducing folic acid levels, alone or in combination with vitamin B12, may have such a beneficial effect, should possibly be investigated.

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